

UREASE DISTRIBUTION IN PLANTS

by

Sam Granick



REPRINTS OF PUBLICATIONS FROM A THESIS SUBMITTED IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY IN THE UNIVERSITY OF MICHIGAN, JUNE, 1936.

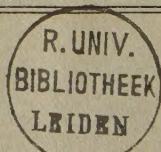
Part I. Urease Distribution in Plants: General Methods. Plant Physiology.
April, 1937.

1. Introduction	471
2. Histological urease determinations	
(a) Indicator method	472
Indicators used	473
Permeability of cells to indicators and urea	473
Buffer capacity of cells and reagents	474
Time factor	475
Method for preparation of tissue sections	475
(b) Lake method; principles and applications	475
3. Quantitative urease determinations	476
Factors concerned in method	477
Effect of drying of tissues on urease activity	478
Effect of macerating	478
Effect of ethyl ether, ethyl chlorhydrin, sodium chloride, high urea concentrations	479
Effect of urea concentrations	479
Temperature. Reaction time	480
Rate of urease inactivation; effect of hydrogen sulfide	480
Hydrogen-ion concentration	481
Sampling of plant material	481
4. Discussion and conclusions	482
5. Summary	485
6. Literature cited	486

Part II. Urease Distribution in *Canavalia ensiformis*. Plant Physiology.
July, 1937.

1. Introduction	601
2. Urease distribution in:—	
Cotyledons	602

Table of Contents continued on inside back cover.



UREASE DISTRIBUTION IN PLANTS: GENERAL METHODS¹

S A M G R A N I C K ²

Introduction

Although numerous studies have been made on enzymes with the purpose of elucidating the intermediary metabolism of various substances, such as the fats, carbohydrates, and proteins, the metabolic changes undergone by the enzymes, that is, their synthesis, storage, and decomposition have received scant attention. Of course the metabolism of an enzyme could be discussed only superficially until a few years ago, because its composition was totally unknown. The discovery by SUMNER (22) that an enzyme, urease, possessing the properties of a globulin, could be obtained in a crystalline state, marked the beginning of a new era in enzyme chemistry. Likewise, the methods developed by LINDERSTRØM-LANG and HOLTER (13) for the application of quantitative histochemistry to enzymes, have led away from a study of gross structures to a more intimate study of tissues and even of cells.

The purpose of the present investigation was to follow the changes in the content and distribution of urease throughout the life cycles of the two leguminous plants which, so far as known, are the richest in their content of this enzyme, namely, the soy bean and the jack bean. The most sensitive tests for most proteins are valueless below a dilution of one part in 10,000. Urease can be detected in much greater dilutions than this. Advantage was taken of the catalytic nature of the enzyme to select certain histological staining reagents which would reveal the presence of the enzyme directly within the cells. By means of these reagents it is possible to study the changes of urease activity taking place during the histological development of the plant. Since the number of cells per organ (for example, the leaf) does not change greatly after an early stage in the development of that organ, one can study, by analyzing organs of different ages, the progressive changes in urease content of cells of increasing age.

It was hoped that the data would not only reveal the course of urease metabolism but would also clarify a number of other problems as well. Considering urease as a protein, one can follow the metabolism of a protein of specific constitution throughout the life history of a plant. If the assumption be made that the main portion of the protoplasmic framework of a cell

¹ Paper no. 610 from the Department of Botany and Herbarium, University of Michigan.

This is the first of a series of three papers on urease distribution in *Canavalia ensiformis* and *Soja max.* More extensive data and discussion of this subject may be found in the writer's thesis in the University of Michigan library.

² Newcombe Fellow in Plant Physiology, University of Michigan.

consists of proteins, it might be possible, by following the changes in urease activity of the cells under various environmental conditions, to get some information about the changes occurring in the protoplasmic framework. Also, since urease has been found in plants of every division of the plant kingdom (4, 27), it becomes interesting to determine what fundamental relation the enzyme has to the nitrogenous metabolism of the plant.

The reaction catalyzed by urease, as shown by MACK and VILLARS (15) and by later investigators, is the hydrolysis of urea to ammonium carbamate. Urea itself is very slightly ionized. Ammonium carbamate is alkaline; it is unstable, especially in acid solutions, and undergoes spontaneous hydrolysis to ammonium carbonate. Two general methods have been used in this investigation: a histological method which depends on the increase in alkalinity as urea is hydrolyzed by urease; and a quantitative method based on the determination of the ammonia produced.

Histological urease determinations

An attempt was made to find a method which would give direct visual evidence of the exact location of urease in the tissues. WAGENAAR (25), SEN (20), and WASICKY and KRACH (27) used a number of reagents. The last-named investigators found that reagents for the determination of NH_4^+ such as Nessler's reagent, chloroplatinic acid, etc., were much less sensitive than pH indicators, and suggested the use of an alcoholic haematoxylin solution as a suitable indicator. After testing a number of methods, the writer came to the same conclusion as WASICKY and KRACH, namely, that the change of hydrogen-ion concentration was the most sensitive method upon which to base the detection of urease.

Two histological methods were used, the indicator and the lake methods, both of which depend upon the detection of urease by an increase in alkalinity.

INDICATOR METHOD

A number of factors which would influence the sensitivity and accuracy of the indicator method had to be considered. These may best be appreciated by imagining an enzyme point in a cell at which urea molecules are undergoing decomposition. The first molecules of ammonium hydroxide released at this point will react with various buffers in the immediate vicinity of the enzyme point. This ammonium hydroxide will therefore not show up as a change in hydrogen-ion concentration and the indicator will not be affected. After sufficient ammonia has been produced the pH of that region will shift toward the alkaline side. The rapidity of the pH change will then partially depend on buffer capacity. It is readily seen that the most sensitive indicator to choose will be one whose midpoint (pK) will be that of the

hydrogen-ion concentration of the cells. It is therefore necessary to know the hydrogen-ion concentration of the tissues examined. The series of indicators recommended by SMALL (21) was found suitable for an approximate determination of the pH of the tissues.

INDICATORS USED.—Because of their visually favorable change in color from yellow at the acid end to dark blue at the alkaline end, the indicators brom-cresol purple, brom-thymol blue, brom-cresol green, etc., were used in a 0.2 per cent. aqueous solution to detect enzymic action. They were adjusted to their midpoint with 0.05N acid or alkali. Although these indicators showed the presence of the slightest traces of enzyme their colors were not intense, but quite diffuse, so that only a vague notion could be obtained as to the enzyme location. Haematoxylin, on the other hand, was found to be excellent for the purpose. Saturated aqueous solutions of haematoxylin were prepared fresh every three or four days since changes take place in this indicator on standing. It is yellow at the acid end and intensely red at the alkaline end of its range, with a midpoint around pH 6.5. Besides showing a marked color change with change in pH, the dye in its alkaline form appears to be quite localized in a tissue. It was found upon further study that the permeability of the cells is much less to alkaline haematoxylin than to the neutral or acid haematoxylin. The yellow-colored (acid) haematoxylin could be washed out readily with water, without as readily removing the red-colored haematoxylin.

PERMEABILITY OF CELLS TO INDICATORS AND UREA.—If one is to judge the location of the enzyme by a change in color of an indicator within the cells, one must know that the indicator and urea solutions have penetrated all the different cells of a tissue section. The penetration of the indicator is quite readily determined by noting its distribution. Generally, the dye colors all of the cells of a tissue section. However, in certain instances, as, for example, in sections of the collet region of jack bean seedlings, some pith cells appear to be less readily permeable to the dye than their neighbors. In such instances, the dye solution is allowed to remain on the section for a few minutes longer until these cells also are colored.

It has generally been considered that cells are readily permeable to urea. HÖFLER and STIEGLER (8), HÖFLER and WEBER (9), and WEBER (28) have shown, however, that the penetration of urea into the cells of various tissues of the same plant and even of the same tissue was different; permeability toward urea was also found to differ with the age and the activity of the cells. By treating cells of various tissues with 20 per cent. ethyl alcohol in order to destroy the plasma membranes and permit unhindered diffusion of urea into all of the cells, it was found that the controls and alcohol-treated sections showed no marked differences in permeability toward urea. In a few instances the alcohol-treated sections appeared to contain slightly less

enzyme, but the distribution even in these sections was the same as in the controls. Perhaps even the least permeable cells allowed sufficient urea to enter from the aqueous urea solution. SMALL (21) reports an increased permeability and outward diffusion of salts from cells treated with 20 per cent. alcohol. The enzyme apparently is not rapidly destroyed in 20 per cent. alcohol. Indeed, sections of jack bean cotyledons immersed for a few minutes in a 95 per cent. alcoholic solution still possessed active urease.

BUFFER CAPACITY OF CELLS AND REAGENTS.—As already indicated, it is necessary to take into account the buffer capacity of the cells studied because, if ammonia is released at the enzyme points, the buffers of the cell will neutralize the ammonia; the rate of indicator change toward the alkaline side will therefore depend upon the buffer capacity of the cell. Although one cell may have a greater enzyme content than another, yet the amount of enzyme in the first cell may be masked by its greater buffer capacity. Likewise, it is possible that a cell may possess both the enzyme and a high buffer capacity, whereas a neighboring cell may contain no enzyme but have a low buffer capacity; in this instance ammonia would diffuse to the neighboring cell containing no enzyme and change the indicator to the alkaline side and apparently indicate the presence of the enzyme. It was found that the meristematic tissues, including the cambium, are most highly buffered, next come the smaller parenchyma cells, then the larger parenchyma cells of the cortex and pith, and finally the xylem cells.

At first glance it would seem that, because of the impossibility of controlling the tissue buffers, the indicator method would be useless for the exact localization of the enzyme in specific cells and tissues. Fortunately, this is not generally so. In sections of the apical bud, where buffer capacity must be considered, it is found that the apical primordium and the axillary primordia, which are the most highly buffered tissues, are also the tissues containing the most enzyme. On the other hand, the buffer capacity in the cambium does not permit a comparison of the enzyme content of that tissue with the content of neighboring parenchyma cells. This is true in the stem below the third node (first node above the cotyledonary node) of the seedlings. In these parts, the enzyme content of the pith and cortex is relatively high, but none can be detected with certainty in the cambium or its derivatives. Above the third node, however, the pith and cortical parenchyma contain no enzyme detectable by the indicator method, and the cambium also gives a negative reaction. That the cambium and its derivatives contain no urease is a generalization that may be made, while keeping the limitations of the indicator method strictly in mind.

Since the enzyme is detected by a change in the hydrogen-ion concentration, it is essential that the reagents used should also have little or no buffering power. Determinations of the buffer capacity of the 0.2 per cent.

dye solutions and the 1 per cent. urea solution showed it to be very slight in these solutions. As contrasted with the buffer capacity of any of the parenchyma cells examined, that of the indicators and of the urea solutions is negligible.

TIME FACTOR.—It is evident that if urease is permitted to act for a longer time more ammonia will be formed and the tissue will become more alkaline. The maximum time for observation is limited by the following considerations: In the first place, carbonic acid is constantly being produced, and neutralizing a portion of the ammonia released. In certain tissues, the enzyme content is so slight that more CO_2 than ammonia is produced and the acidity of the tissue increases, as has been determined in a few experiments with a sensitive glass electrode apparatus. In the second place, the enzyme appears to be slowly inactivated. Thirty minutes was found to be the maximum time that could be used for these histological determinations.

TISSUE SECTIONS.—For approximate estimates of the enzyme, sections were made with a hand razor. If delicate structures were to be examined or accurate comparisons of sections were to be made, a freezing microtome was used at first. It was soon found, however, that small pieces could be imbedded in paraffin of low melting-point and rapidly cooled, without appreciable destruction of the enzyme. The time, from removing the material from the plant until the section was ready for study under the microscope, varied between 15 and 20 minutes.

LAKE METHOD

Although a particular indicator will permit the detection of urease by the first appearance of color change, the ammonia generated at the enzyme points diffuses so rapidly into the surrounding cells that the color of the alkaline indicator becomes quite diffuse. The ammonia may also invade tissues containing no enzyme and the indicator test may then give spurious results. It is therefore necessary, for sharp localization of the enzyme, to secure a reagent which will form a non-diffusible precipitate at the enzyme points. At the same time it is desirable to poison the enzyme points in order to prevent ammonia from being continuously liberated and invading the neighboring tissues. It is necessary, moreover, that the precipitate be readily visible within the cells. Of the large number of reactions tested, the formation of a lake (an adsorption complex of a dye with the hydroxide of a heavy metal) satisfies these three requirements.

The inactivation of a urea solution with various metals has been studied by SCHMIDT (19), and more recently by JACOBY (11). The following was found to be the order of poisoning effectiveness: $\text{Cu}^{++} > \text{Hg}^{++} > \text{Ag}^+ > \text{Ni}^{++} > \text{Fe}^{+++} > \text{Zn}^{++} > \text{Pb}^{++}$. The last elements had only comparatively slight effect on urease activity.

The formation of metallic hydroxides at various hydrogen-ion concentrations has been studied by BRITON (3) and others. They find that the hydrogen-ion concentrations at which the hydroxide will form is a characteristic of that metal. For example, Ni^{++} will form its hydroxide when added to a solution whose hydrogen-ion concentration is pH 6.7 or above. The hydroxides of the heavy metals are generally very insoluble gelatinous precipitates which would be difficult to discern when formed within the cells. With the proper dye adsorbed, it is possible to detect traces of metallic hydroxides.

The application of the principle outlined is best illustrated by a specific example. Microtome sections of an apical meristem are treated with aqueous haematoxylin. This is allowed to remain on the sections for a few minutes until it has penetrated all of the cells. Excess fluid is drained off and a drop of a 1 per cent. urea solution and a drop of 0.05 M NiCl_2 are now added. A cover slip is placed over the sections, excess fluid is drained off, and the slide is examined immediately with a microscope to note the distribution of the deep blue color of the nickel hydroxide-haematoxylin lake.

The order of adding the reagents depends on the amount of enzyme present. If the enzyme is highly concentrated, as it is in the mature cotyledons, it is advisable to use a solution of nickel chloride and in addition a trace of cupric or mercuric chloride to poison the enzyme more rapidly. This solution is added immediately after the urea. If, on the other hand, the enzyme content is very low, it may be desirable to add a very dilute nickel chloride solution some time after the urea solution is added. The enzyme action will convert the yellow haematoxylin to a red-purple color. On addition of the dilute nickel chloride solution, this color will become deep blue and quite sharply delimited from the yellow of the surrounding tissues.

Quantitative urease determinations

An excellent discussion of various quantitative methods for the determination of urease is given by EULER (4). After several methods had been tested, a colorimetric method was chosen. The ammonia produced by a piece of tissue containing urease, after the latter has acted on a urea solution for a certain time, is driven over with a rapid current of air into an acid solution in which the ammonia is determined. With some modifications the method is essentially that described by VAN SLYKE and CULLEN (29).

A weighed portion of fresh tissue is finely ground in a porcelain mortar with 1 to 2 cc. of M phosphate buffer (pH 7.2), with quartz sand when necessary. The mixture is transferred to a volumetric flask of appropriate capacity, made up to volume with distilled water at 30° C., and the flask placed in the water bath (30° C.). Into each of the several reaction tubes

are pipetted 1 cc. of phosphate buffer, 1 cc. of freshly prepared 2 M urea solution, and the requisite amount of distilled water. The amount of distilled water is so chosen that the final volume of solution in the reaction tube is exactly 10 cc. The tubes are also placed in the water bath. After they have come to the temperature of the bath, an aliquot of tissue solution is pipetted into each tube. The time from the beginning of grinding until the tissue is added to the reaction tube varies between 10 and 15 minutes. Exactly 30 minutes (stop watch) after the tissue is placed in the tube the urease is inactivated by the addition of 0.5 cc. of 2.5 N H_2SO_4 . The acid solution serves a twofold purpose. It not only inactivates the enzyme solution completely, but also decomposes the ammonium carbamate which was produced by the hydrolysis of urea. (Alkali does not inactivate urease completely.) After 5 minutes, the reaction tube is placed in the aeration apparatus. A few drops of caprylic alcohol and 5 gm. of anhydrous K_2CO_3 are rapidly added, the tubes stoppered, and suction from the water pump is applied. Control tubes containing tissue but no urea are treated in all respects like those described above. After all the ammonia has been driven over into the tube containing acid, it is determined colorimetrically with a Duboseq colorimeter. If the ammonia content is above 0.05 mg., the customary Nessler's reagent is used. For determinations of less than 0.05 mg. NH_3 , the sodium phenate reagent as modified by VAN SLYKE and HILLER (30) was used.

The sum of the ammonia present in the tissues and in the reagents, subtracted from the total ammonia values obtained, gives the ammonia produced through the decomposition of urea by the urease of the tissues.

Using a maximum of 0.5 gm. of tissue and a time-period of 30 minutes for reaction the method permitted the determination of a wide range of enzyme activity. In actual practice, the enzyme values ranged from 30 U.U. down to 0.0001 U.U. where *U.U.*, the urease unit, is a quantity of urease which will produce one milligram of NH_3 per minute under the experimental conditions employed.

FACTORS CONCERNED IN QUANTITATIVE UREASE DETERMINATIONS

The quantity of urease is determined indirectly by measuring the rate of formation of ammonia from urea under certain standardized conditions. These conditions were chosen and various other factors examined in relation to their influence on urease activity. Although numerous publications have dealt with the quantitative measurements of the activity of a particular tissue containing an enzyme few bear upon the difficulties that arise in an attempt to make comparisons of the quantity of enzyme in the same tissue at different stages of development, or in different tissues. A number of factors cannot be adequately controlled. However, an approximation to the true values of enzyme activity can be obtained.

A number of methods have been used by investigators to prepare the plant material for enzyme analysis. For determinations of the total enzyme content it is obviously useless to extract the enzyme, since extraction is never complete; it is different for different materials; and during extraction inactivation of the enzyme occurs.

EFFECT OF DRYING.—It is important to learn whether tissues may be dried without causing changes in enzyme activity. PETT (17) has studied the effect of drying germinated wheat seeds and concludes that the dipeptidase activity increases on drying. Since he used a glycerol extract of the material for his enzyme determinations, it may be that drying merely increased the amount of enzyme extracted by the glycerol, and not the dipeptidase activity.

In the following experiments the material was spread out on filter paper and allowed to dry at room temperature (approximately 22° C.). The urease content was determined immediately and after several days of drying.

TABLE I
EFFECT OF DRYING ON UREASE ACTIVITY

PLANT MATERIAL	TIME AFTER COLLECTING	MOISTURE	U.U. PER BEAN
		%	
Soy beans, nearly mature (beans removed from pods; 10 used for each determination)	Immediate	61.0	1.01
	1 day	35.2	0.988
	7 days	10.1	0.976

TABLE II
EFFECT OF DRYING ON UREASE ACTIVITY

PLANT MATERIAL	TIME AFTER COLLECTING	U.U. PER GM. OF ORIG- INAL FRESH WEIGHT
Jack bean leaflets (young; 5-8 cm. long; 15 used for each determination)	Immediate	0.0542
	6 days	0.0344

The data in table I show only a slight decrease in urease content during slow drying of nearly mature soy beans. In the young leaflets of jack bean (table II), however, air-drying decreased the urease activity 37 per cent. Because of these results it was decided that all analyses should be made on fresh tissues.

EFFECT OF MACERATING.—To determine what effect crushing and macerating the tissues would have on urease activity, opposite leaflets (with petiolules removed) of jack bean plants were used. These leaflets possess numerous small stomata on the under surface but none on their upper surface. Whole leaflets, leaflets cut into 5-mm. squares, and macerated leaflets were compared

as to their urease activity. It was found that with a 0.2 M urea solution the whole leaf showed an activity one-thirtieth that of the macerated tissue. The 5-mm. squares of leaflets had an intermediate value. Infiltration of whole leaflets with urea by means of suction, and increasing the length of time for reaction on the urea solution to 60 minutes, increased the indicated urease values of whole leaflets to one-fourth that of macerated leaflets. It appeared probable that the lower value of urease activity, if whole leaflets were used, was because of the inability of the urea solution to come into contact with all of the available enzyme points in the cells. It was thought possible that the permeability of the plasma membranes might be increased by the addition of 1 and 2 per cent. ethyl ether or ethyl chlorhydrin to the urea solution. Determinations on macerated, as well as on whole tissue, showed, however, that these chemicals, at the concentrations used, inhibited urease activity from 15 to 25 per cent. No increase in urease activity was observed with leaf-tissue mash suspended in 5 per cent. sodium chloride. Another series of experiments was performed to determine whether very high urea concentrations would not give values for urease activity of the whole leaf approximating those of macerated leaves. Using 1 to 4 M urea solutions, it was found that the activity of urease in whole leaves, especially at the highest urea concentrations, approximated that of macerated leaves. It may be concluded that maceration of the jack bean leaves causes no detectable inactivation of urease. In this connection it is interesting to note that LINDERSTRÖM-LANG and HOLTER (13) report no difference in the activity of peptidase present in whole sections of barley roots as compared with crushed sections.

UREA CONCENTRATION.—An attempt was made to choose a sufficiently high urea concentration so that the amount of decomposition produced in a given time would be independent of the concentration. The data of VAN SLYKE and CULLEN (29) and of LÖVGREN (14) on soy bean extracts indicate that the rate of decomposition does not increase above a concentration of 2 M urea, although the increase above 0.2 M is slight. Results were obtained on soy bean cotyledons that were in accord with the findings of these investigators; that is, there was no appreciable increase in the decomposition rate above 0.2 M concentration of urea. A concentration of 0.2 M was then chosen for the quantitative investigations. It is a convenient concentration to handle, because a 2 M urea solution can be made up, and 1 cc. of this pipetted into a reaction tube, tissue mash and buffer added, and the whole finally diluted to 10 cc.

It was later found that the conclusion arrived at for the urea concentration of soy bean cotyledons does not hold for jack bean leaf tissue. Even above a 2.1 M substrate concentration there still is an appreciable increase in the rate of urea decomposition. With 0.1 M urea solution, 0.61 mg. NH_3 was produced; with 0.7 M urea solution, 0.68 mg. NH_3 ; with 2.1 M urea

solution, 0.72 mg. NH_3 ; and with 3.5 M urea solution, 0.76 mg. NH_3 . Obviously, one cannot speak of a maximum concentration above which all the enzyme points are being readily supplied with urea without specifying the tissue-preparation used. One may conclude that, if different tissues or plant parts, such as roots and leaves, are to be compared as to total urease content, an error of 25 per cent. may arise merely because of insufficient urea concentration; that when the same tissues or plant parts are being compared this error is eliminated.

The *temperature* chosen was 30° C. This is sufficiently high temperature to effect rapid enzymic hydrolysis, but not high enough for inactivation of the enzyme to become an important factor.

The *reaction time* was chosen as 30 minutes. This was selected after a number of preliminary trials had been made. Because the rate of inactivation of different enzyme preparations could not be controlled, the shortest time was chosen in which determinations of the lowest enzyme concentrations could be determined accurately. This of course depended on the sensitivity of the method selected for the ammonia determinations.

Some idea of the rate of urease inactivation was obtained from the following experiments. The moment that the maceration of the tissues was begun was taken as zero time. After a thorough grinding and proper dilution, the mash was placed at 30° C. and overlaid with toluene. At various intervals, an aliquot of the mash was taken for analysis, and allowed to react on the urea for 30 minutes at 30° C.

TABLE III
RATE OF INACTIVATION OF MASH CONTAINING UREASE AT 30° C.

MATERIAL USED	TIME AFTER BEGINNING OF MACERATION		U.U. PER GM. FRESH WT.
	<i>min.</i>	<i>hr.</i>	
Soy bean cotyledons (germinated 36 hours)	{ 55		3.92
	{ 115		3.86
	{ 295		3.54
Jack bean leaflets (5-8 cm. long)	{ 49		0.0547
	{ 94		0.0550
	{ 194		0.0603
	{ 361		0.0605
		21	0.0574
		140	0.0069
Jack bean leaflets (5-8 cm. long; dried at room temperature 6 days)	{ 66		0.0344
	{ 396		0.0041

From table III it is seen that the decrease of urease activity in the soy bean cotyledons is negligible during the first few hours. It is interesting to note that the mash, made from fresh leaflets of jack bean, actually increases in urease activity during the first three hours. Here again the change of activity, during the first hour after addition of urea, is negligible. With leaflets of jack bean, dried at room temperature for six days, the rate of inactivation appears to be especially marked; no change in urease activity was observed on the addition of 0.5 cc. of saturated aqueous H_2S to the reaction tubes containing an aliquot of mash. These experiments indicate that two simultaneous processes are going on: an inactivation of the enzyme by denaturation, coagulation, or poisoning; and a dispersion, peptization, or activation of the enzyme.

The *hydrogen-ion concentration* of pH 7.2, which was produced by a phosphate buffer, was used for the urease determinations. LÖVGREN (14), using phosphate buffers, reported an optimum pH between 7.2 to 7.8 for soy bean extracts. HOWELL and SUMNER (10) found that the urease activity depended on the type of buffer and on the concentration of urea, the pH optimum increasing with decreasing urea. BACH (1) found an optimum hydrogen-ion concentration of pH 8.6 for a urease preparation extracted from an *Aspergillus*. It is quite possible that the optimum hydrogen-ion concentration for a urease preparation may depend to some extent on its concomitant impurities, as has already been found for certain enzymes. No experiments were conducted to determine whether the hydrogen-ion concentration optimum is different for different structures of the same plant. With the dipeptidases of the wheat grain PETT (17) found about the same pH-activity curve for all portions of the wheat grain that were studied.

Since ammonia is produced during urea hydrolysis and the solution becomes alkaline, it is necessary to have a sufficient amount of buffer present to prevent any marked change in the hydrogen-ion concentration. In this investigation, a quantity of enzyme material has been taken which will generally produce less than 1 mg. of NH_3 in 30 minutes. A concentration of 0.1 M phosphate buffer (prepared from Na_2HPO_4 and KH_2PO_4) was used, which when present in the enzyme substrate mixture was found to keep the hydrogen-ion concentration from increasing more than three-tenths of a pH unit.

The *recovery of ammonia with the aeration apparatus* was determined, using standard solutions of ammonium sulphate, and also using mash plus standard ammonium sulphate solutions. Although 90 per cent. of the ammonia was recovered after 30 minutes of aeration at room temperature, recovery was complete only after two hours of aeration. The presence of mashed tissue did not interfere with recovery.

SAMPLING OF PLANT MATERIAL.—Analyses were made on the basis of the fresh weight of plant organs or parts of organs. The organs were removed

from the plants in the morning, then weighed and analyzed immediately. For each analysis generally ten organs were selected from ten different plants. The tissues were ground and duplicate determinations were run on aliquots of the mash, including also a control for the free ammonia present in the mash. In selecting samples for analysis, an attempt was made to choose plants that were similar in number and length of leaves and internodes. Plants either more or less vigorous than the average were discarded.

Discussion and conclusions

It is necessary to clarify the term "quantitative" in reference to enzyme determinations. An enzyme is detected only through the catalysis of a more or less specific reaction. This criterion is, however, not sufficient to establish the absence or the total quantity of enzyme, but merely the amount of "active" enzyme present.

An extensive review by LÖVGREN (14) of the inactivation of urease by various substances indicates that inactivation, in most instances, can readily be accounted for by a denaturation or coagulation of the protein. As for activation by specific substances, WALDSCHMIDT-LEITZ and STEIGERWALD (26) report that urease activation with glycine or hydrocyanic acid is observed only with crude enzyme preparations, and not with purified or crystalline enzymes. There is some evidence (2, 5, 7) that oxidation inactivates and reduction reactivates urease but the meaning of this is not clear.

The data at present available on crystalline hydrolytic enzymes, including urease, favor preponderantly the contention (24) that these enzymes are proteins containing in their make-up, and holding by chemical linkages, peculiar arrangements of atoms which constitute the active groups. Since urease can be detected only in its active state, that is, by the number of active groups with which the urea molecules can come in contact, it becomes important to examine under what conditions and to what extent these groups are available for action.

In the cells, proteins are present in various states of aggregation, some protein molecules being monomolecularly dispersed, others clustered into larger groups, and still others aggregated into ergastic protein materials such as the globoids, aleurone grains, and crystals. The protein urease likewise can be considered to be present in the cells in these various states of aggregation. GRABAR and REIGERT (6) have recently investigated the activity of urease as related to particle size. Using four different urease preparations they found, by means of membranes of graded porosity, that the particles of urease were in different states of aggregation. Crystalline urease in aqueous solution was the most homogeneous preparation examined, and had dimensions near those of serum globulin. On digestion of the urease preparations with trypsin it was found that those particles small enough to pass through pores 15 m μ in diameter possessed no urease activity. Under the same

conditions serum albumin passed through pores 30 μ in diameter but not through 9 μ pores. Crystalline urease was the most active preparation. From these data, it appears that the activity of a urease preparation is at its maximum when the urease particles are of the dimensions of serum globulin particles (mol. wt. 150,000); presumably the urea molecules are now able to come into contact with all the available active groups of the urease particles. The work of KIRK and SUMNER (12) may be cited as evidence that the decrease of urease activity occurs with agglomeration of urease particles. These workers noted that the inhibiting effect of anti-urease (antibody) on urease consists, largely but not entirely, in decreasing the dispersion of urease.

From the above, it may be seen that when larger groups of urease molecules, as in the form of ergastic materials in the cell, are dispersed, an apparent synthesis may occur. If urease is decomposed proteolytically below a certain molecular weight there will be a loss of urease activity; but there may also be a decrease in urease activity or apparent decomposition when the enzyme becomes denatured, agglomerated, or stored in some form in which it is not readily dispersed. The urease that is detected is, in the main, in a highly dispersed condition. The data on the rate of inactivation of mash of jack bean leaf show an increase in urease activity during the first three hours after maceration, indicating that more of the active groups have become available than are being removed by autolysis or denaturization.

There is no way at present of determining the total quantity of enzyme present (16, 18). The methods used in this investigation have been chosen to determine as nearly as possible the relative amounts of available active groups present in the cells at the time of examination. In all instances, where comparisons could be made of the histological and the quantitative methods with the same tissue, it was found that the results obtained with both methods checked each other nicely. One may infer, therefore, that in the macerated tissue, the enzyme does not differ in activity to any extent from the enzyme in the cells of tissue sections. The experiments in which "whole" and "macerated" leaf tissues were compared showed that macerating caused no detectable inactivation of the enzyme. Further experiments (table III) indicate that urease inactivation within the first 30 minutes after crushing the tissue is slight or negligible; and this has been the length of time chosen for the enzyme to act on urea.

Although, with the cotyledonary tissue of soy bean, there is no increase in the rate of urea decomposition above 0.2 M urea concentration, this is not true for the tissue of the jack bean leaf. In this leaf tissue, there still appears to be an appreciable increase in the decomposition rate of urea even above a 2.1 M concentration. This may be another instance of making the active groups of urease available, since it is known that urea in high aqueous concentrations has a powerful solvent action on many proteins, and appears to split certain

protein molecules. Edestin, for example, is split into particles whose molecular weight is about one-fourth of that determined by SVEDBERG for edestin in neutral salt solutions. For convenience, a concentration of 0.2 M urea was used in these determinations. When different plant structures are to be compared as to total urease content, an error of 25 per cent. may arise because of an insufficient urea concentration; when the same tissues or plant structures are being compared, however, this error is eliminated. Fortunately, the differences of urease content of different structures are much greater than 25 per cent. so that this factor does not influence any of the conclusions that have been drawn from the data.

Although the limit of sensitivity of the quantitative method is 0.0001 U.U. per gram of fresh weight of tissue (where a urease unit, U.U., is that quantity of urease which will produce 1 mg. of ammonia per minute), the histological method is not as sensitive. From comparative determinations it has been found that the indicator method cannot detect urease in tissues having a lower activity than 0.02 U.U. per gram fresh weight.

By making certain assumptions,³ it may be calculated that there are about 25 urease molecules contained in a cell, at the limits of sensitivity of the quantitative method. Since the indicator method is only one-two hundredth as sensitive as the quantitative method, at least 5000 urease molecules must be present in a cell in order to change the indicator color. It is therefore impossible to determine the positions of the enzyme in the cells by the indicator method unless the urease molecules are sharply localized in specific regions.

Summary

1. Two methods for the determination of urease are described. The first, a histological method, depends on detecting the increase in alkalinity of the cells as urea is being hydrolyzed by the urease present in the cells. This increase in alkalinity is made evident either by the use of a suitable pH indicator or by the formation of a lake. The second, a quantitative method, depends on the determination of the ammonia produced when urea is hydrolyzed by the enzyme.

2. The following factors were considered in applying the histological method: The hydrogen-ion concentration of the tissue; the suitability of various indicators; the permeability of cells to indicator dyes and to urea; the buffer capacities of cells and reagents; the time factor for the method; and the preparation of tissue sections for analysis. In the lake method, reagents were chosen which would form insoluble and highly colored precipitates at the enzyme points.

³ Assumptions: Dimensions of a parenchyma cell $20 \times 50 \times 50 \mu$; density of cell 1.0; there is one active group per urease molecule; urease has a molecular weight of 150,000; 1 gm. of crystalline urease can produce 26,000 mg. NH_3 per minute, which is the highest activity reported by SUMNER (23).

3. The following factors were considered in applying the quantitative method to various tissues: Effect of drying the tissues; effect of macerating the tissues; effect of various urea concentrations on the maximum urease activity; effect of the temperature; effect of the time; the rate of inactivation of urease; the effect of the hydrogen-ion concentration; the choice and concentration of the buffer; the recovery of ammonia by aeration; and the sampling of tissues for analysis.

4. A concept of urease activity, based on the number of available active groups of urease, is discussed.

The writer wishes to express his gratitude for the many helpful suggestions and the kindly criticisms of Professor F. G. GUSTAFSON throughout the course of this investigation.

UNIVERSITY OF MICHIGAN
ANN ARBOR, MICHIGAN

LITERATURE CITED

1. BACH, D. Evolution de l'uréase dans les cultures de *l'Aspergillus niger*. Bull. Soc. Chim. Biol. **11**: 1007-1015. 1929.
2. BERSIN, T. Thiolverbindungen und Enzyme. Ergebn. Enzymf. **4**: 68-101. 1935.
3. BRITON, H. T. S. Electrometric studies of the precipitation of hydroxides. Jour. Chem. Soc. London **127**: 2110-2159. 1925.
4. EULER, H. Chemie der Enzyme. II Teil, 2 Abschnitt: 334-370. München. 1927.
5. FISHER, H. The relation between activity of urease and the oxidation-reduction potential. Biochem. Jour. **28**: 406-410. 1934.
6. GRABAR, P., and REIGERT, A. Ultrafiltration de l'uréase sur des membranes de porosités graduées. Compt. Rend. Soc. Biol. **117**: 712-714. 1934. **119**: 1004-1007. 1935. Compt. Rend. Acad. Sci. (Paris) **200**: 1795-1797. 1935.
7. HELLERMAN, L., PERKINS, MARIE, and CLARK, W. Urease activity as influenced by oxidation and reduction. Proc. Nat. Acad. Sci. **19**: 855-860. 1933.
8. HÖFLER, K., and STIEGLER, A. Ein auffälliger Permeabilitätsversuch in Harnstofflösung. Ber. d. bot. Ges. **39**: 157-164. 1921.
9. ———, and WEBER, F. Die Wirkung der Äthernarkose auf die Harnstoffpermeabilität von Pflanzenzellen. Jahrb. wiss. Bot. **65**: 643-737. 1926.
10. HOWELL, S. F., and SUMNER, J. B. The specific effects of buffers upon urease activity. Jour. Biol. Chem. **104**: 619-626. 1934.
11. JACOBY, M. Zur Kenntnis der Wirkungen der Metalle auf Fermente. Biochem. Zeitschr. **259**: 211-222. 1933.

12. KIRK, J., and SUMNER, J. B. Immunological identity of soy and jack bean urease. *Proc. Soc. Exp. Biol. & Med.* **29**: 712-713. 1932.
13. LINDERSTRØM-LANG, K., and HOLTER, H. Beiträge zur enzymatischen Histochemie. II. Über die Peptidaseverteilung in Wurzel und Blattkeim des Malzkornes. *Zeitschr. physiol. Chem.* **204**: 15-53. 1932.
14. LÖVGREN, S. Studien über die Urease. *Biochem. Zeitschr.* **119**: 215-293. 1921.
15. MACK, E., and VILLARS, D. S. Synthesis of urea with the enzyme urease. Action of urease in the decomposition of urea. *Jour. Amer. Chem. Soc.* **45**: 501-505, 505-510. 1923.
16. OPARIN, A. Die Wirkung der Fermente in der lebenden Zelle. *Ergebn. Enzymf.* **3**: 51-72. 1934.
17. PETT, L. B. Studies on the distribution of enzymes in dormant and germinating wheat seeds. I. Dipeptidase and protease. II. Lipase. *Biochem. Jour.* **29**: 1898-1904. 1935.
18. PRZYLECKI, S. Über die intracelluläre Regulierung der Enzymreaktionen mit besonderer Berücksichtigung der Amylasewirkung. *Ergebn. Enzymf.* **4**: 111-146. 1935.
19. SCHMIDT, E. G. The inactivation of urease. *Jour. Biol. Chem.* **78**: 53-61. 1928.
20. SEN, P. B. A method of locating urease within a tissue by a microchemical method. *Indian Jour. Med. Res.* **18**: 79-82. 1930-1931.
21. SMALL, J. Hydrogen-ion concentration in plant cells and tissues. *Protoplasma-Monographien II*. Berlin. 1929.
22. SUMNER, J. B. The isolation and crystallization of the enzyme urease. *Jour. Biol. Chem.* **69**: 435-441. 1926.
23. ————. Crystalline urease. *Ergebn. Enzymf.* **1**: 295-301. 1932.
24. TAUBER, H. The chemical nature of enzymes. *Chem. Rev.* **15**: 99-121. 1934.
25. WAGENAAR, M. Bijdrage tot de kennis der localisatie van urease in sojaboonen. *Pharm. Weekbl.* **61**: 535-542. 1924.
26. WALDSCHMIDT-LEITZ, E., and STEIGERWALD, F. Über den proteolytischen Abbau krystallisierter Urease. *Zeitschr. physiol. Chem.* **206**: 133-136. 1932.
27. WASICKY, R., and KRACH, S. Zur verbreitung der Urease im Pflanzenreich. *Osterr. bot. Zeitschr.* **77**: 271-279. 1928.
28. WEBER, F. Harnstoff-permeabilität ungleich alter Stomatazellen. *Protoplasma* **14**: 75-82. 1931.
29. VAN SLYKE, D. D., and CULLEN, G. E. A permanent preparation of urease, and its use in the determination of urea. *Jour. Biol. Chem.* **19**: 211-228. 1914.
30. ————, and HOLLER, ALMA. Determination of ammonia in blood. *Jour. Biol. Chem.* **102**: 499-504. 1933.

UREASE DISTRIBUTION IN *CANAVALIA ENSIFORMIS*¹

SAM GRANICK²
(WITH TEN FIGURES)

Introduction

In this account the distribution of urease, as studied with the histological and quantitative methods which were described in the first paper of this series (2), will be discussed under the subheadings of the various plant structures. The definition of some of the terms used are as follows:

Urease activity is expressed in urease units. A urease unit (U.U.) is a quantity of urease which will produce 1 mg. of ammonia per minute when acting upon urea under the experimental conditions employed (2). The indicator method is not as sensitive as the quantitative method, and tissues containing less than 0.02 U.U. per gram of fresh weight are reported as negative with this method. Tissues containing less than 0.0001 U.U. are reported as negative with the quantitative method.

The *hypocotyl* is that part of the axis between the cotyledonary node and the external collet.

The *external collet* is the region below the hypocotyl, bearing a considerable tuft of lateral roots.

The *first internode* is the hypocotyl.³

The *first node* is the cotyledonary node.

The *second node* is that part of the stem axis from which the first foliage leaves arise.

Canavalia ensiformis or jack bean is an annual legume usually bushy and erect, 1 to 2 meters high; the tips of the branches inclined to twine; peduncles stout, 10 to 20 flowered; pods linear, 25 to 30 cm. long; seeds ellipsoid, shiny white, $22 \times 14 \times 8$ mm. The plants grown in the greenhouse at Ann Arbor were 3 to 4 meters high and spindling. The distinctive taxonomical description of *Canavalia ensiformis* is given by PIPER (5). The gross morphology of the plant closely resembles that of *Phaseolus vulgaris* whose anatomy has recently been studied by DOUTT (1).

¹ Second of a series of three papers on urease distribution in *Canavalia ensiformis* and *Soja max.* The reader is referred to the thesis available at the University of Michigan library for more extensive data on urease in the jack bean, for descriptions of the relevant anatomical and embryological features of the plant, and for the literature on the presence of urease in various species of the plant kingdom.

Paper no. 611 from the Department of Botany and Herbarium, University of Michigan.

² Newcombe Fellow in Plant Physiology.

³ This convenient designation, used solely for the purpose of this paper, is not technically correct according to morphological terminology which would consider the first internode as the region between the cotyledonary node and the first foliage leaves.

The bulk of the seed of jack bean consists of cotyledons which are made up largely of parenchyma cells with thickened hemicellulose walls possessing numerous tiny pits. SUMNER (6) estimates that the bean contains 0.12 to 0.06 per cent. urease and considers the crystalline urease to be globulin, because it is insoluble in the absence of neutral salts in the vicinity of its isoelectric point. About 25 per cent. of the air-dried seed is protein (4).

Experimentation

COTYLEDONS

Employing the histological urease methods, examination has shown that the cotyledons contain more urease than any other tissues of the plant. Studies of the seeds after two days of germination in sphagnum revealed that the enzyme concentration was greatest in the subepidermal cells, and especially in the bundle sheaths, and phloem tissue, that is, in those cells of the cotyledons containing the densest cytoplasm (fig. 1). The bundles of the cotyledons immediately adjacent to and merging with the conducting bundles of the hypocotyl appeared to contain less urease than the other bundles of the cotyledons. This may be due to a more rapid elongation of the bundles adjacent to the cotyledons, the enzyme thus becoming more diffuse, or it may be due to decomposition of the enzyme in the bundles. As germination proceeds, urease decreases rapidly in the epidermis and in the two to three layers of subepidermal parenchyma cells. The enzyme disappears from some parenchyma cells more rapidly than from others. No relation could be noted between the enzyme content of the cells close to the bundles, and those more or less isolated from the bundles. This suggests that there is no transport of active urease to the bundles. The cotyledons which fell off after 20 days still contained relatively large quantities of urease. Sectioning at this stage revealed that the cells were thin-walled with large vacuoles, and contained numerous chloroplasts but little starch.

TABLE I
UREASE CONTENT OF COTYLEDONS OF JACK BEAN SEEDLINGS

DAYS AFTER PLANTING	FRESH WT. PER COTYLEDON	DRY WT. PER COTYLEDON	U.U. PER GM. FRESH WT.	U.U. PER COTYLEDON
<i>days</i>	<i>gm.</i>	<i>gm.</i>		
0	0.651	0.560		
1	1.05	0.561	31.2	32.8
3	1.25	0.532	25.9	32.5
5	1.15	0.456	24.4	28.0
7	1.26	0.377	12.6	15.9
10	1.17		11.5	13.5
20*	0.483		1.08	0.523

* The cotyledons had just fallen off; they were quite thick, yet flaccid.

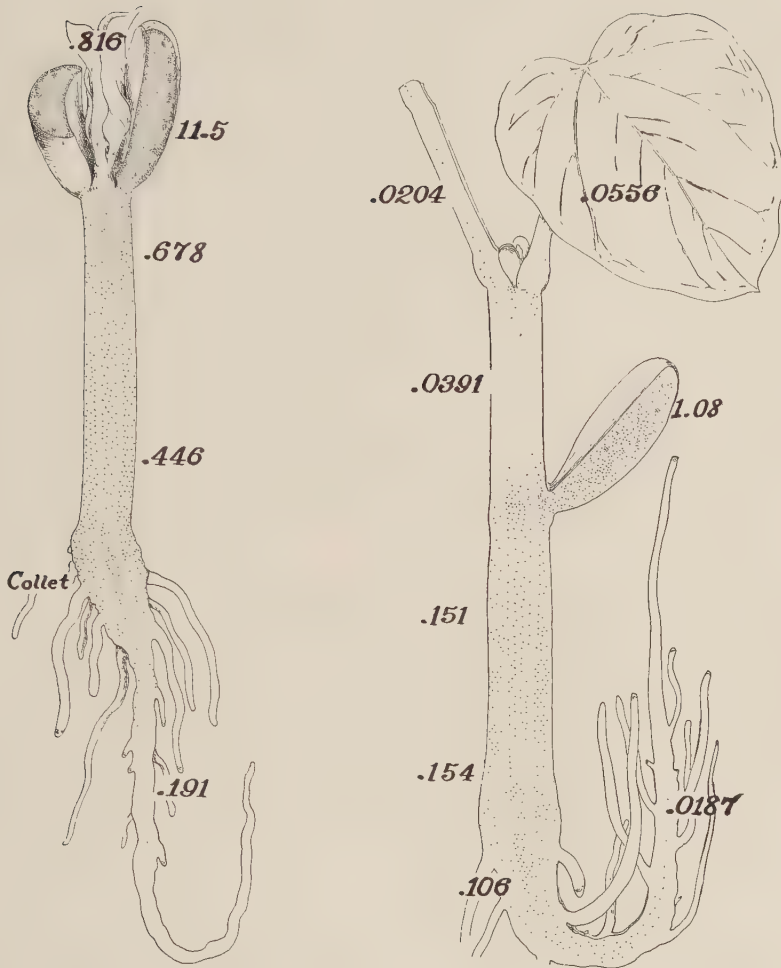


FIG. 1. Composite picture of urease distribution in the various structures of jack bean seedlings 10 and 20 days old. The figures opposite the various regions of the seedling represent the number of urease units per gram of fresh weight of the region, as determined by the quantitative method. Stippling indicates the relative urease concentration as revealed by the histological methods.

Quantitative studies were made of the urease content of cotyledons during the development of the seedlings. It will be noted from table I that the urease content of the cotyledon decreases rapidly as the seedling becomes older. The data are interpreted as indicating, not that the enzyme is gradually inactivated by poisons or other inactivators, nor that it is transported intact to other tissues, but that it is broken down just as are other storage proteins of these cells, into some soluble compounds which are then

transported to other plant parts. This viewpoint will be discussed later in more detail.

RADICLE

In the radicle 1 cm. long (fig 2.) the histological reagent shows urease to be present in all of its parts except the primary xylem elements. The

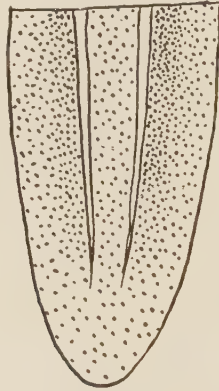


FIG. 2. Urease concentration in a radicle 1 cm. long.

inner cortical cells contain the most enzyme, the outer cortical cells and pith less enzyme, and the cells of the radicle tip still less. The dermatogen appears to contain little or no enzyme; it is, however, slightly more acid (pH 4.4) than the other tissues. The weak indicator-change in the dermatogen may possibly be due to the fact that more acid has to be neutralized before the indicator shows an alkaline reaction than in the other tissues. Therefore, a positive statement cannot be made concerning its relative urease content.

Cross-sections of a 3-cm. radicle reveal a decrease of urease in the region in which cell elongation has occurred, that is, at approximately 2 cm. distal to the tip. At this level there is less enzyme in the pith than in the cortex. After four days' germination the radicle has attained a length of 5 cm. and the collet region with its tuft of secondary roots has appeared.

COLLET

The collet region was made the subject of particular study, since it is in this region that the various stages of lateral root differentiation can most readily be obtained. In 5-day-old seedlings urease tests showed that the enzyme was most concentrated in the elongated narrow cells of the outer cortex (pH 4.0); the inner cortex and pith (pH 5.5) contained slightly less enzyme. It was difficult to judge the urease content in lateral root primordia because of the relatively high enzyme activity of the parenchyma cells of

the collet. The lateral root primordia arising endogenously from the pericycle cells opposite the protoxylem always contained less urease than did the parenchyma cells. By removing the larger lateral roots (1 to 3 cm.) and examining them separately, it was found that they contained very little enzyme except in the apical meristems. In 4-week-old seedlings, sections of the collet indicated a low urease activity, the pith containing somewhat more urease than the cortex. In 7-week-old plants the pith gave a barely perceptible test for urease; the cortex gave no test.

Root

In the 5-cm. radicle, a distinction between root and hypocotyl is already evident, owing to the appearance of a lateral tuft of roots at the collet region. An examination was made of the urease content at different levels of the primary root. The enzyme was present in the apical meristem. It was absent in the cambium and its derivatives of secondary phloem and xylem which were already differentiated in the region 2 cm. above the tip. The

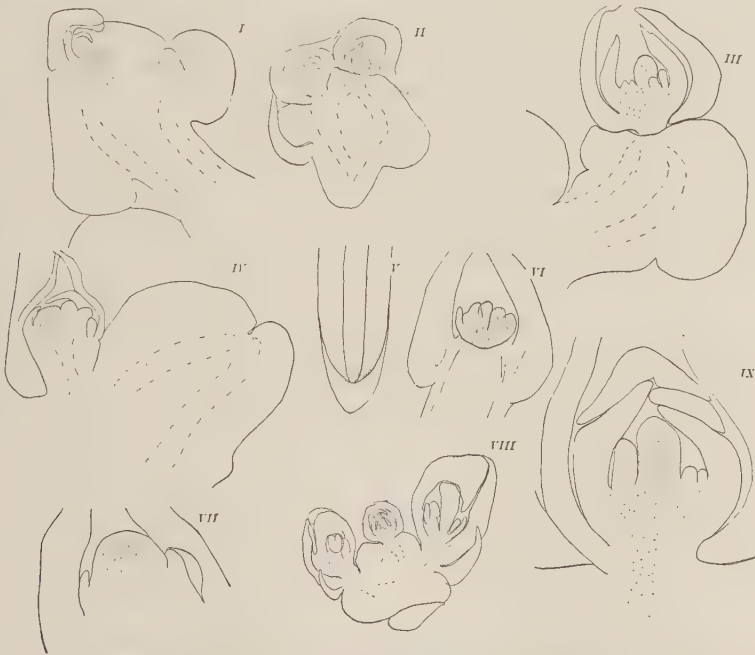


FIG. 3. Urease concentration and distribution in meristems of jack bean (as shown by intensity of stippling in camera lucida drawings).

I and II, sections made through different levels of the same pedicellar gland, showing urease in the very young flower meristems and in the gland.

III, IV, VIII, IX, urease distribution in later stages of flower differentiation.

V, distribution of urease in a longitudinal section of root tip.

VI, urease content of a cluster of promeristems of a leaf axil.

VII, distribution of urease in an apical vegetative meristem.

parenchyma cells of the cortex and pith, however, contained urease throughout the entire length of the root.

Seven days after planting, the root had increased to 20 cm. At this stage, the root tip contained very small quantities of urease. Roots, sectioned longitudinally in paraffin or on a freezing microtome, showed a positive reaction for urease in only about 5 per cent. of the root tips examined. The enzyme in these root tips was strictly delimited to the meristematic region (fig. 3, V). Whole root tips also were examined. The following modified procedure gave good results. A number of root tips were placed on a slide and treated with haematoxylin, in 20 per cent. alcohol, for 15 minutes. The dye penetrated into the cells and the root tips became yellow. (A few root tips, which appeared to be injured or sickly, turned red, that is, they were more alkaline than the rest. These were discarded.) Excess dye was drained off, 1 per cent. urea solution was added, and a cover slip applied. After 2 to 10 minutes, a red color appeared which varied from a very weak positive to a weak positive test. Every root showed this red region at the meristematic zone, and not far above it. The variations in urease distribution in the root tips examined by this method are shown in figure 4. The maximum urease

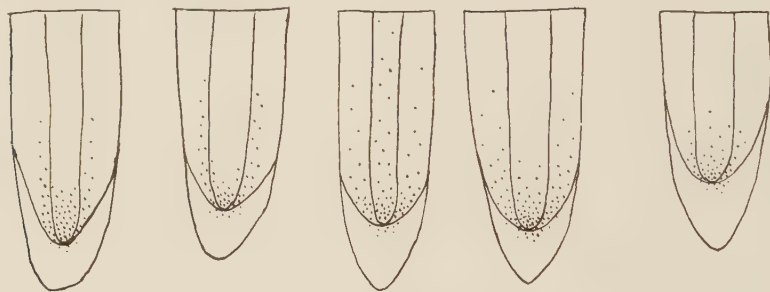


FIG. 4. Urease distribution in root tips.

content is in the region of actively dividing cells. In some root tips there is more urease in the periblem than in the plerome, and in others the opposite is true.

A large number of root tips from 13-week-old plants were examined. Most of these root tips gave a negative reaction for urease with the staining reagents. However, those that were positive showed that urease was located only in the meristematic region.

The studies of LINDERSTRØM-LANG and HOLTER (3), on the quantitative distribution of peptidase in the germinating barley root, led them to conclude that the maximum peptidase activity was related to the region of cell elongation and not to the region of rapid cell division. The experiments on urease relate the maximum urease concentration to the region of rapid cell division.

The data for the quantitative urease determinations on the roots of the jack bean are given in table II. It is seen from these figures that the urease

TABLE II
UREASE CONTENT OF ROOTS OF JACK BEAN SEEDLINGS

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.		U.U. PER UNIT STRUCTURE	
			+ COLLET	- COLLET	+ COLLET	- COLLET
<i>days</i>		<i>gm.</i>				
1	radicle	0.025	19.2		0.480	
3	radicle	0.300	2.94		0.824	
5	root + collet	0.310	0.434		0.134	
7	root + collet	0.788	0.169		0.133	
10	root + collet	0.645	0.191		0.123	
20	root	0.625		0.0187		0.0117
.....	root + collet	0.997	0.0515		0.0514	
28	root	2.37		0.0114		0.0271
40	root	3.31		0.0079		0.0263
.....	root + collet	3.77	0.0105		0.0396	

activity per gram of fresh weight decreases with increasing age of the roots. This is to be expected if one assumes: that (a) the cells of the root, back of the region of cell division, do not possess any marked ability to synthesize or elaborate urease, in which case only a dilution of the enzyme already present in the cell would occur during cell elongation; that (b) owing to the partial sloughing off of the outer cortical cells of the root, and a partial disintegration of the pith, these urease-containing cells are destroyed and the enzyme in them is likewise destroyed; that (c) owing to the increase of dead xylem cells, the percentage of living cells in the root decreases and therefore the urease activity per unit of fresh weight also decreases. From the figures on the urease activity per unit tissue of "root + collet," it is seen that a definite decrease of urease must occur even in the early stages of root formation. This decrease of the enzyme is not readily evident during the first 10 days after germination because of the rapid increase in the number of new roots, and consequently of young cells containing urease. After this time, the number of roots increases slowly and the value for total urease drops off rapidly. The data also indicate that during the first 10 days the collet region possesses approximately five times as much urease activity as the roots.

HYPOCOTYL

The hypocotyl of the 5-day-old seedling possesses a high urease content throughout its entire length of 4 cm. The enzyme appears mainly in the parenchyma cells of the cortex and pith. The epidermis and the small subepidermal parenchyma cells contain little of the enzyme. No urease could be detected, with the histological reagents, in the cambium or its derivatives.

The decrease of urease activity follows the elongation of the hypocotyl as would be expected if dilution of the enzyme were taking place. The stippling in figures 1 and 5 indicates the relative urease activity of the 10-, 20-, 28-, and 40-day-old seedlings. The enzyme does not continue to decrease indefinitely, for in the 12-week-old plant the hypocotyl still gives a weak reaction in the pith and cortex with the histological reagent. The content of urease in the hypocotyl is always highest just below the cotyledons and just above the collet region, disappearing most slowly from the collet region. Whether the cells in these regions remain small, or whether less urease is catabolized, has not been determined.

From the quantitative data in table III, it may be observed that the change in urease activity is quite marked, decreasing from 0.777 U.U. per

TABLE III
UREASE CONTENT OF HYPOCOTYL (FIRST INTERNODE)

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE	
				ENTIRE HYPOCOTYL	PARTS OF HYPOCOTYL
<i>days</i>		<i>gm.</i>			
1	Radicle	0.025	19.2	0.480	
3	Radicle	0.300	2.94	0.824	
5	Hypocotyl 3.5 cm.	0.480	1.62	0.777	
10	Hypocotyl 7 cm.	0.921	0.561	0.518	
	Upper half	0.460	0.678		0.312
	Lower half	0.461	0.446		0.206
20	Hypocotyl 12 cm.	1.802	0.153	0.275	
	Upper half	0.857	0.151		0.129
	Lower half	0.945	0.154		0.148
28	Hypocotyl	1.401	0.0550	0.0770	
40	Hypocotyl	1.708	0.0317	0.0541	

hypocotyl in the 5-day-old seedling to 0.0541 U.U. in the 40-day-old plant. The changes in structure of the hypocotyl with age, such as the conversion of some parenchyma cells into pericyclic and collenchymatous tissue, etc., do not account for the marked decrease in total urease content of the hypocotyl. A decrease in the enzyme content of the parenchyma cells is evident.

STEM

The apex of the stem was made the subject of a detailed study since this material contains, within the short space of 3 mm., the embryonic meristem and the differentiating tissues. Longitudinal sections, cut at 35 and 60 μ were tested with the urease reagents. These showed that urease was located in greatest quantity in the primordial meristem. With the enlargement of the isodiametric cells of the meristem to form cortex and pith, the enzyme rapidly decreased in concentration, being absent in some stem tips at the region where metaxylem elements are already visible.

A few photomicrographs (fig. 6) were taken of sections of apical buds treated with the haematoxylin-nickel reagent for urease. In the presence of ammonia, a dark purple lake is formed which shows up black in the photographs. The photographs do not indicate the actual sharpness with which the enzyme may be localized by this method, since it was necessary to permit the development of a more intense and therefore a somewhat more diffuse color in order properly to photograph the sections. Figure 6 A shows two meristems containing much urease, while figure 6 B shows two meristems one of which contains more enzyme than the other.

There were great variations in the enzyme content of the various apical and axillary buds examined. Sections of some buds gave no urease test. Most of them gave only a weak reaction (fig. 3, VII). A few gave fairly strong reactions. The weakly reacting sections evidenced limitation of urease to the promeristem of the tip and to the promeristems in the leaf axils. With sections of higher urease activity both the promeristems and the procambium possessed a relatively high urease content; furthermore, the cortex, the pith, or both together, gave a positive urease reaction extending well into the elongation-region (3 mm. from the tip). The procambium strands gave weaker reactions as they differentiated, and by the time a few metaxylem elements had appeared they gave no reaction for urease. In these buds it was difficult to judge the urease content of the cambium or its derivatives because of the urease activity of the neighboring parenchyma cells. In cross-sections of young stem tissue 15 mm. below the apical bud, the enzyme was just faintly detectable only in the pith.

A comparison was made between the urease activity of the axillary and the apical buds taken from the same plant. It was found that some axillary buds contained more urease than the apical buds, or *vice versa*. It also appeared that apical buds of vigorously growing shoots possessed a somewhat higher urease activity than apical buds of slower-growing shoots.

The quantitative data on the urease content of the stem internodes (table IV) indicate that the U.U. per gram of fresh weight values are highest in the uppermost, and therefore the youngest, internodes. This is to be expected

TABLE IV
UREASE CONTENT OF DIFFERENT INTERNODES OF THE STEM

INTERNODE	AVERAGE LENGTH	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER INTERNODE
	<i>cm.</i>	<i>gm.</i>		
6	3 (leaf bud)	0.0088	0.066	0.0006
5	5	0.150	0.0127	0.0019
4	10	0.585	0.0059	0.0034
3	9	0.644	0.0049	0.0028

since the histological studies on the stem tip showed that the youngest cells possessed the highest urease activity. The urease activity per gram of fresh weight in the lower internodes decreases progressively with age owing to the dilution of the enzyme and probably also to a catabolism of the enzyme.

EPICOTYL

The region above the cotyledonary node, *i.e.*, the epicotyl, is considered separately from the other internodes because it is one of the organs which is already developed in the seed and these organs have been found to possess very high urease contents. Sections of the young, second internode show

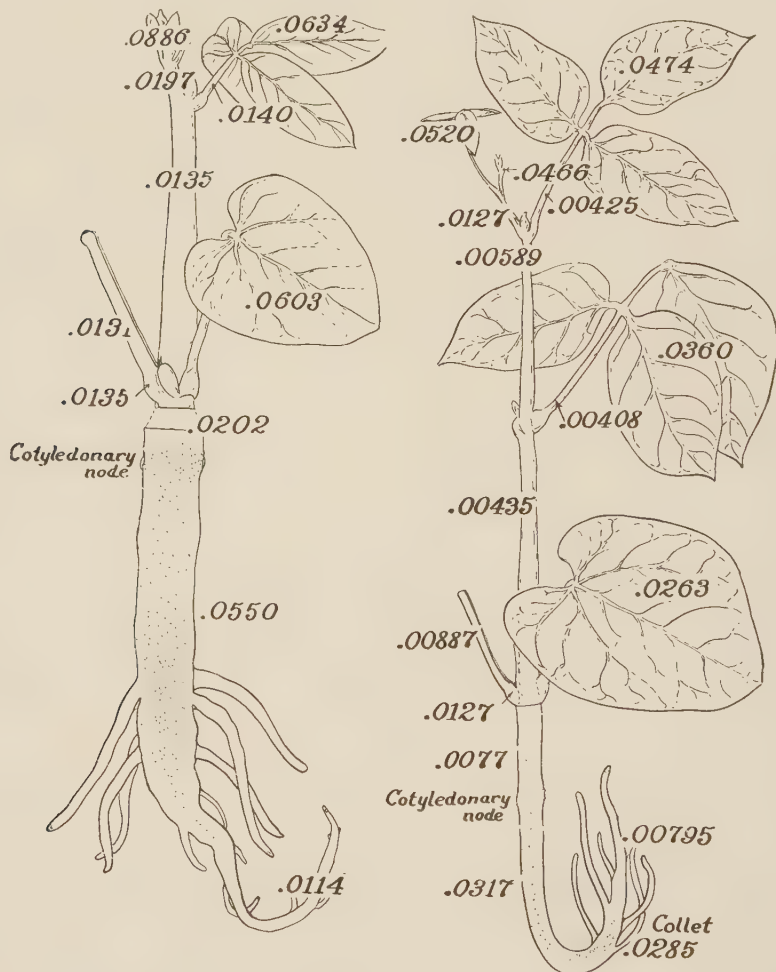


FIG. 5. Composite picture of urease distribution in the various structures of the jack bean seedlings 28 and 40 days old. (See legend of fig. 1.)

active urease in the pith and cortex. As the internode increases in length, the enzyme concentration decreases until after 28 days (fig. 5) it is detectable only close to the cotyledonary node. From the quantitative data in table V it may be seen that the urease content per epicotyl is highest when the plant is 15 days old. The most rapid elongation has occurred between the tenth and fifteenth day. After this period of rapid elongation the urease

TABLE V
UREASE CONTENT OF EPICOTYL (SECOND INTERNODE)

AGE OF PLANT	LENGTH	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER EPICOTYL
<i>days</i>	<i>cm.</i>	<i>gm.</i>		
3	0.9 (plumule)	0.0120	1.35	0.0162
15	3.5	0.180	0.222	0.0401
20	5.0	0.412	0.0391	0.0161
28	5.5	0.512	0.0202	0.0103
40	5.2	0.675	0.0077	0.0052

content per epicotyl drops off markedly. Urease activity per gram of fresh weight is greatest in the meristematic tissue of the young 9-mm. plumule and decreases rapidly as these cells elongate and age. This same sequence of urease changes with age has also been found in the other stem internodes.

LEAVES

Examination of longitudinal sections of the differentiating leaves of a large number of axillary and apical buds revealed that the very young leaves enveloping the growing point contain urease, at times more, and at other times less than the growing point itself. The enzyme content of the buds as a whole varied greatly, some containing much, others little urease. No reason has as yet been found for this variation. The procambial tissue at the base of the developing leaf gave a strong reaction for urease. Even at a slightly later stage, when a portion of the procambium strands had differentiated into definite protoxylem elements, the content of urease was still high in the protophloem and procambium. No differences in urease activity were noted among the various parenchyma cells.

In a study of leaflets 10 mm. long it was found that the veins gave a stronger urease reaction than did the other portions of the leaf blade. Examination of the veins indicated that the enzyme was limited to the large area of parenchyma cells below the vascular bundles, and to a lesser degree to the bundle sheath, the reaction of the phloem being doubtful.

Sections of leaflets 4 cm. long from the first leaf below the apical bud again showed that the cortical cells of the midrib and of the other main veins contained more urease than did the other cells of the leaf blade. The strong-

est urease reaction of the leaf was given by the cortical cells of the midrib at the base of the leaf. No difference could be noted in the urease content of parenchyma cells throughout the length of the leaf. In cross-sections, the enzyme appeared to be more concentrated in the palisade cells than in the

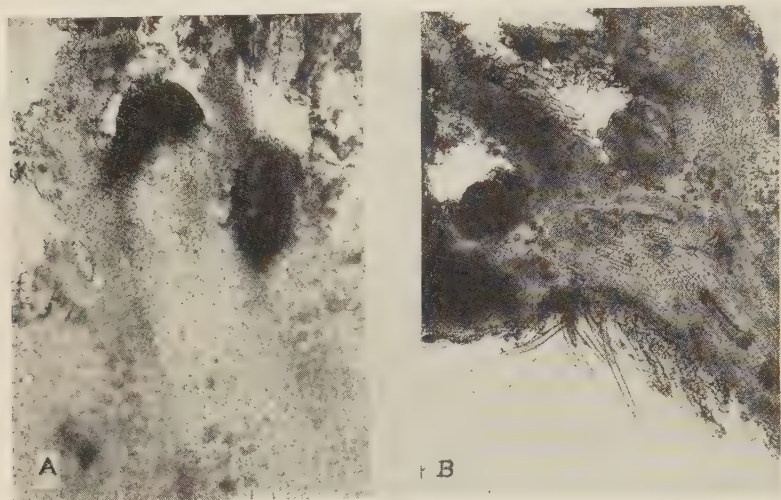


FIG. 6. Urease distribution in meristems of apical buds of jack bean. Darker areas indicate higher urease content.

spongy mesophyll. This may be due, however, to the compactness of the palisade cells which, under the conditions of the test, would effect a more rapid increase in alkalinity than would the loose mesophyll cells, even though the urease contents were the same, cell for cell. The epidermal cells appeared to contain less urease than the mesophyll cells. In leaflets 12 cm. long, no indications of the enzyme distribution could be obtained because of the low urease content.

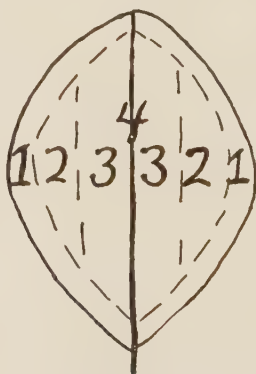


FIG. 7. Leaflet showing place from which samples were taken for urease determinations.

Quantitative determinations were made on the distribution of urease in different portions of leaflets 12 cm. long and 5.3 cm. wide. Portions of these nearly mature leaflets were taken as shown in figure 7. It is seen from table VI that the data on U.U. per gram of fresh weight indicate that urease

TABLE VI
UREASE CONTENT OF PORTIONS OF LEAFLET (FIG. 4)

No.	PART OF LEAFLET	FRESH WT.	U.U. PER GM. FRESH WT.
		<i>gm.</i>	
1	Outermost 5-mm. edge	0.720	0.0310
2	10-mm. middle strip	0.998	0.0345
3	10-mm. inner strip	1.161	0.0342
4	Midrib	0.365	0.0123

activity is about the same throughout the various portions of the lamina. The enzyme content of the outermost edge of the lamina is somewhat less than that of the other portions of the lamina. This may or may not be due to the fewer parenchyma cells as compared to epidermal cells in this portion of the lamina. The midrib of the leaflet contains only about one-third as much urease per gram of fresh weight as the other parts. As has been mentioned above, the parenchyma cells in the veins contain much urease. The relatively low figure for U.U. per gram of fresh weight in the midrib is due to the large proportion of primary xylem and of collenchyma cells which contain little or no urease.

A quantitative study was made on the amounts of enzyme in leaves of different sizes. Under similar environmental conditions the young, actively growing ternate leaves of the same size showed remarkably similar values for urease content. Apparently the position of the leaf on the stem makes little difference. Indeed, it was possible to predict approximately the total urease content of any size of actively growing leaf because of this constancy of relation. From table VII it is evident that the youngest tissues possess the highest urease activity per gram of fresh weight. As the leaves grow older, these values decline. The figures for "U.U. per unit structure" show clearly that a definite synthesis of urease has taken place during the expansion of the cells, and long after cell division has ceased in the young leaf. Contrasting the leaf 6.5 cm. long and the fully expanded leaf 15 cm. long, it is seen that the urease content has increased from 0.037 to 0.149 U.U. per leaf.

PLUMULE LEAVES

The first leaves (plumule leaves) to appear after the germination of the seed are a pair of simple, opposite, cordate leaves located at the second node. These were laid down in their embryonic condition within the developing

seed. As noted previously, the tissues formed in the developing seed possess, in their early stages, a higher urease activity than those which develop from the meristems after germination. Because of this higher content, detailed studies of urease distribution in these leaves could be made with the histological reagents.

TABLE VII
UREASE CONTENT OF DEVELOPING TERNATE LEAVES

STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
	<i>gm.</i>		
1-mm. apical bud	0.0016	0.08	0.0001
3-mm. apical bud	0.0028	0.05	0.0001
17-mm. apical bud at 6th node	0.0265	0.046	0.0012
3.5-mm. leaf	0.0088	0.066	0.0006
1.4-cm. leaf at 5th node	0.0385	0.053	0.0020
6.5-cm. leaf blade at 4th node	0.780	0.047	0.0370
15.0-cm. leaf blade at 3d node	4.115	0.036	0.149
Petiole of leaf at 4th node	0.603	0.0042	0.0026
Petiole of leaf at 3d node	0.579	0.0041	0.0024

Sections of the young plumule leaf (3 mm. long) showed a strong urease reaction throughout the leaf, the veins containing slightly less enzyme than the embryonic parenchyma cells. In the slightly older leaf (8 mm. long) the large cortical cells of the midrib contained less urease than did the embryonic parenchyma and epidermal cells. In the leaves 20 mm. long, the picture of urease distribution was similar to that obtained with the trifoliate leaves, that is, the palisade cells gave the strongest urease reaction, the mesophyll cells less, and the epidermal cells still less. Epidermal layers, stripped from the leaves and examined for urease, gave indications of the presence of only traces of enzyme. Sections through a leaf 8 cm. long from a 17-day-old plant showed that the greatest urease concentration was present in the parenchyma cells of the midrib lying below the vascular bundles. The urease reaction was especially strong at the base of the leaf, *i.e.*, in the secondary pulvinus. In the petiole proper, only a slight urease activity was noted. At the base of the petiole, in the pulvinus, only the lower portion of the cortex contained the enzyme (fig. 5).

Determinations were also made of the quantity of urease in the plumule leaves at various stages of their development. The leaves follow the same trends of urease content as those noted for the stem internodes and the roots (table VIII). The youngest leaves have the highest urease activity per gram of fresh weight and as the leaves become older the urease activity per gram of fresh weight diminishes rapidly. The urease content per unit of organ shows that a definite synthesis of the enzyme takes place until elongation of

TABLE VIII
UREASE CONTENT OF THE PAIR OF PLUMULE LEAVES

AGE OF PLANT	STRUCTURES	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
<i>days</i>		<i>gm.</i>		
10	2-cm. leaves	0.0101	0.816	0.083
20	8.5-cm. leaf blades	2.066	0.0556	0.115
28	13-cm. leaf blades	4.990	0.0603	0.306
40	13-cm. leaf blades	5.730	0.0263	0.150
28	13-cm. leaf blades:			
	main veins	0.850	0.0377	0.032
	blade minus main veins	4.14	0.0662	0.274
20	Both petioles	0.538	0.0204	0.0110
28	Both petioles	0.662	0.0132	0.0088
40	Both petioles	0.783	0.0098	0.0078

the cells has practically ceased. From that time on inactivation or catabolism of urease occurs. The petioles undergo these changes more rapidly than do the leaves. The urease content of the plumule leaves in their adolescent stage (approximately 2 cm. long) is much higher (0.81 U.U. per gm. fresh weight) than that of the ternate leaves of similar size (0.05 U.U. per gm. fresh weight). Stipules of the second node were also examined, and, as would be expected, they undergo changes in urease content similar to the leaves.

FLOWER AND FRUIT

The flowers arise on knoblike protuberances, the pedicellar glands, which may be borne sessile in the axil of a leaf in close proximity to the axillary vegetative bud; or they may arise from a stalk, the peduncle, which may grow out to a length of 10 to 15 cm. and upon this peduncle may be borne one or more pedicellar glands, each of which may bear 3 to 5 flowers (fig. 3, IV, VIII).

The distribution of urease was studied in sections of flower primordia, in a manner similar to that described for the vegetative primordia. A photomicrograph of a longitudinal section, through a nodal region, stained with the lake reagent for urease, is shown in figure 8. It will be noted that there are several promeristems present in a very early stage of differentiation, all of which stain darker than the neighboring tissues, and thus contain more urease. It is probable that the cluster of three promeristems at the left are the "anlage" of the flower primordia and that the promeristem at the right will differentiate into a vegetative primordium. Another view of a cluster of promeristems in a leaf axil may be seen in figure 3, VI, where urease concentration is indicated by the intensity of stippling.

Urease distribution in later stages of flower differentiation is illustrated in figure 3. Figure 3, VIII shows a section through a pedicellar gland bear-

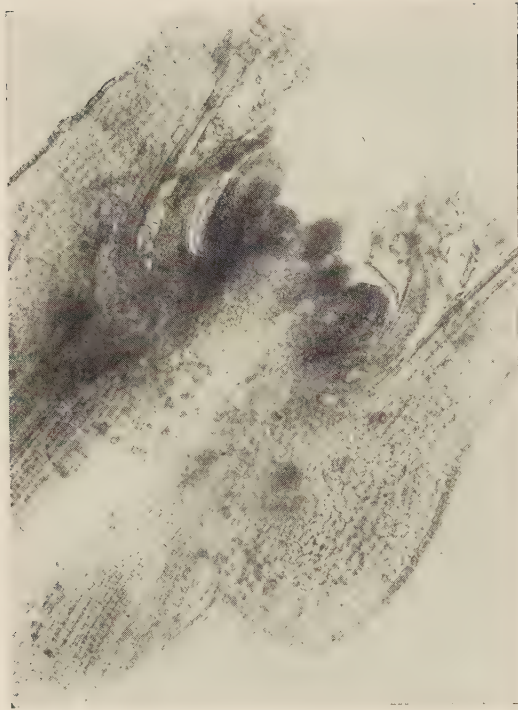


FIG. 8. Longitudinal section of axillary meristems showing the distribution of urease.

ing three flower primordia. Figure 3, I and II, are of sections made through different levels of the same pedicellar gland. They indicate that a relatively high urease activity extends from the flower promeristems into the regions of the pedicellar gland where the cells are still small and active. Later stages of flower differentiation are represented by figure 3, IV, III, and IX. From the numerous observations made on flower primordia, it may be said that, in general, urease is relatively highest in concentration in the flower promeristems; in the differentiating flower it is highest in the primordia of the pistil and the cells connecting the pistil with the pedicellar gland; it is less in the stamen primordia, and least in the primordia of the calyx and corolla.

No urease can be detected in the peduncles with the indicator reagents. A quantitative determination on peduncles varying from 1 to 9 cm. in length gave an average urease activity of 0.0165 U.U. per gram of fresh weight (table IX).

The pedicellar glands are made up of a spongy mass of parenchyma cells. Only those regions of the parenchyma which join or abut on the flower primordia give a faintly positive urease reaction with the histological re-

TABLE IX
UREASE CONTENT OF FLOWER BUDS

STRUCTURES	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
Peduncles (1-9 cm. long)		0.0165	
Pedicellar glands		0.0199	
Tip of raceme; 12 flower buds each less than 2 mm. (av. wt. 0.0064 gm.)	0.0765	0.0542	0.00034 (average)
5-mm. flower bud	0.0144	0.0241	0.00035
13-mm. flower bud	0.152	0.00745	0.00112
14-mm. flower bud (just opening)	0.210	0.00683	0.00143

agents. The total urease activity of these glands is relatively low (0.0199 U.U. per gram fresh weight). From the pedicellar glands arise the flower primordia which contain a fair amount of urease (0.0542 U.U. per gm. fresh weight). As growth and differentiation of these cells continue, urease activity decreases (table IX), until, in the flower bud which is just opening, it is down to 0.0068 U.U. per gram of fresh weight. Although the urease activity per gram of fresh weight has decreased, there has been a definite synthesis of urease per flower bud.

When the flower is mature the pollen grains possess a low but definite urease activity. The pistil at this stage also has a low urease activity (0.0028 U.U. per gm. fresh weight).

Since the endosperm contains 3n or 33 chromosomes, it was considered especially interesting to determine the urease activity of this tissue. Sections of a bean 10 mm. long treated with a urease reagent showed that urease, if present in the endosperm, was below that concentration which could be detected by the histological method. It was however found that the endosperm could be readily removed *in toto* from the embryo sac. A number of endosperms were therefore removed and placed in small test-tubes and urea and indicator added until the total volume was 0.20 cc. Appropriate controls were also run. The endosperm tissue was not crushed. After one hour a definite positive indication of the presence of urease in the endosperm was observed. It is estimated that the endosperm had an approximate urease activity of 0.005 U.U. per gram of fresh weight. This same procedure was used in determining the presence of urease in mature pollen grains.

In a seed, 10 mm. long, the radicle of the embryo is somewhat less than 1 mm. The radicle at this stage possesses a definite but low urease activity. The cotyledons, consisting of small thin-walled cells, also possess a low urease activity. The inner integument consists of two layers of parenchyma cells and an epidermal layer abutting on the embryo sac. This integument has the highest urease activity of any of the tissues in a seed 10 mm. long, the urease activity being somewhat greater at the chalazal end.

The quantitative data (table X) on the urease content of the maturing bean reveal a steady increase of urease as the bean develops. In the young bean the weight of cotyledon and endosperm is negligible compared to that of the testas, and the values for U.U. per gram of fresh weight and for U.U. per bean up to the stage where the bean is 13 mm. long actually indicate the changes occurring in the testas. The testas pass through the same stages as other structures previously described. There is a decrease of U.U. per gram of fresh weight as the cells of the testa enlarge and mature; however, the urease activity per testa increases until the bean is approximately 15 mm. long and thenceforth decreases as the bean continues to enlarge.

TABLE X
UREASE CONTENT OF THE MATURING BEAN

LENGTH OF BEAN	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
	<i>gm.</i>		
4- 6-mm. bean	0.0117	0.0822	0.00096
7- 9-mm. bean	0.0575	0.0467	0.0027
11-13-mm. bean	0.185	0.0262	0.0048
17-mm. bean	0.648		0.178
Embryo	0.352	0.495	0.174
Testa	0.296	0.0162	0.0048
20-mm. bean	1.080		0.400
Embryo	0.646	0.615	0.397
Testa	0.434	0.0080	0.0035
22-mm. bean minus testa	1.60		2.65
Cotyledons	1.58	1.65	2.61
Radicl and plumule	0.0162	2.75	0.044

As the cells of the cotyledons continue to divide and enlarge in the developing embryo, storage of proteins, starch, and hemicelluloses proceeds vigorously and the urease activity likewise increases very rapidly until the bean has attained its full size. In the radicle and plumule the cells elongate only slightly; there is less storage of carbohydrates than in the cotyledons but the cells accumulate proteins and also increase rapidly in urease activity. From the data in table X it is seen that the urease activity of the radicle and plumule of the mature bean (2.75 U.U. per gm. fresh weight) is even higher than the urease activity of the cotyledons (1.65 U.U. per gm. fresh weight). This dominant synthesis of proteins together with urease in meristematic cells of the embryo has already been noted for the other meristematic tissues of the plant with the exception of the cambium, where no detectable amount of urease is produced.

ENTIRE PLANT

A survey of the changes in the urease content of the jack bean plant as a whole, throughout its life cycle, is given in the graph of figure 9. Total

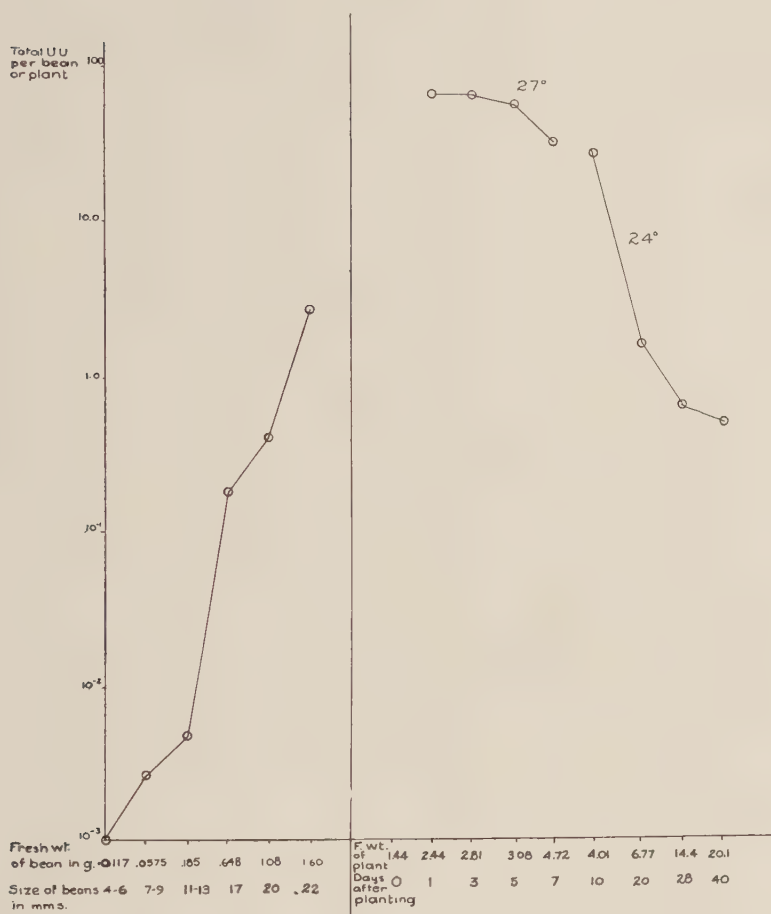


FIG. 9. Graph representing the total urease content of young maturing seeds and of seedlings at various ages after planting. "Total U.U. per bean or plant" is plotted logarithmically along the axis of ordinates. Various stages of seed or plant development are plotted on the axis of abscissas.

urease is plotted logarithmically on the ordinate, and the various stages of development are indicated on the abscissa. As the beans develop and mature in the pods, a rapid synthesis of urease takes place, the urease values increasing over 1000 times when a comparison is made between the urease content of the very young bean (4-6 mm. long) and the nearly mature bean (22 mm. long). The stages of complete maturity and drying were not determined in this plant.

Upon germination the seedlings develop rapidly. Thermograph records were kept and the average temperatures noted at which urease in the seedlings decreases with age, changing from 66 U.U. per seedling after one day's ger-

mination to 0.45 U.U. in the 40-day-old plant. The most rapid decrease in urease content occurred in the 10- to 20-day-old plant. In the 20-day-old plant the cotyledons were about to fall off. Although total urease determinations were not carried out on plants over 40 days of age, it may be said from other data that there will be a slight increase in urease content when more leaves have developed on the plant. Still later, when the seeds begin to form in the pods, a marked increase will take place.

When the data on the urease content of the other plant structures are considered, it becomes evident that figure 9 represents, to a large degree, the urease changes of the cotyledons. The cotyledons contain such preponderating quantities of urease as to overshadow any changes of urease content occurring in the other plant structures. Even when the cotyledons are about to fall off in the 20-day-old plant, they still contain about two-thirds of the total urease of the plant. The marked decrease in urease in the 10- to 20-day-old plant is due to the marked decrease in urease content of the cotyledons. It is interesting to note that even after the cotyledons have fallen off, the urease content per plant still continues to decrease. The decrease in the total amount of urease in the plant is therefore not wholly due to the decrease in the cotyledons.

The distribution of the enzyme in the various tissues of seedlings 10, 20, 28, and 40 days old is illustrated in composite pictures in figures 1 and 5. The stippling in these figures indicates the relative urease concentrations as revealed by histological methods. The figures opposite each structure express the urease units per gram of fresh weight as determined by the quantitative method. A description of the specific urease changes of the individual plant structures will be found under their respective headings in the previous pages.

Discussion

It was realized, only after the analyses were nearing completion, that the urease content in different plant structures, as the internodes, leaves, roots etc., follow similar trends which are related to the ages of these structures. The curves in figure 10, in which the relative ages of the structures are plotted along the abscissa, indicate these general trends. The urease activity per gram of fresh weight (fig. 10 A) is highest when the cells of the young structure are actively dividing, and decreases as the cell of the primary tissues⁴ grow older. The total urease content of a plant region (fig. 10 B) increases rapidly during the period of cell division of the primary tissues, and more slowly during the period of cell elongation until, at about the time

⁴ The term primary tissues, as here used, has reference to those tissues developing from the apical meristems in contrast to the secondary tissues which develop from the cambium.

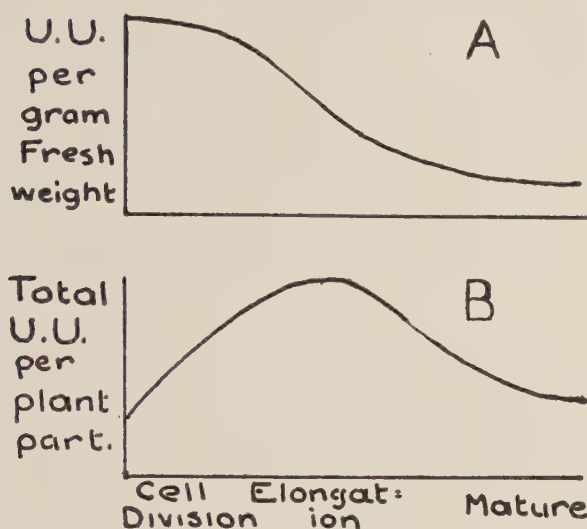


FIG. 10. Changes in urease activity in relation to physiological age.

primary growth has ceased, the plant part contains a maximum urease content.

Consideration of the histology of the seedlings makes it evident that the preponderating cells are the parenchyma cells. These cells originate by the elongation of the isodiametric cells of the apical meristems and appear to be the least differentiated of the cells derived from the meristems. Since it has been found, with the indicator reagents, that neither the cambium nor its derivatives contain perceptible quantities of urease, it becomes apparent that the changes observed in the distribution of urease are really the changes occurring in a single tissue, the parenchyma.

Upon this basis, the urease changes may be related to the aging of parenchyma cells. The urease content per unit volume of cell steadily decreases (fig. 7 A) as a cell ages. The decrease in urease units per gram of fresh weight is especially rapid during the period of cell elongation. After that, there is a slower decrease in the enzyme. This decrease is partially accounted for by the dilution of materials in the cell and by an increase in dry weight due to cellulose deposition, but there is also a definite inactivation or perhaps catabolism of urease taking place especially in the mature cells.

The metabolism of urease in the parenchyma cells may be briefly summarized (fig. 10 B) thus:

1. There is a rapid synthesis of urease in the actively dividing meristematic cells.

2. This synthesis continues during the period of cell elongation although at a decreasing rate, the amount of urease of a cell reaching a maximum at the end of the elongation period.

3. After this period, there is a decomposition of urease which continues down to a certain level.

A discussion of reasons for these changes will appear in the next paper of this series. The parenchyma cells of the root pass through the various growth stages and phases of urease change most rapidly, the stem cells less rapidly and the leaf cells least rapidly.

The cells possessing the densest cytoplasm contain the highest content of urease. This is true for the meristems of the root tip, stem apex, internodes, and flower promeristems. In the cotyledons the subepidermal parenchyma cells, the cells making up the bundle sheaths of the veins, and the young primary phloem cells, which appear to contain the densest cytoplasm, are also richest in urease. The density of cytoplasm is related to its protein content (the major portion of the protein being globulin). If the globulin urease undergoes changes similar to other globulins in synthesis and decomposition, it might be expected that an increase in protein content of the cytoplasm would be accompanied by an increase in urease activity. The data appear to confirm this.

The structures developing from the fertilized egg and laid down in the maturing seed have been treated separately from the structures which are differentiated after the seed germinates because the former possess a urease activity which is higher than that of similar organs developing in the seedling or mature plant. For example, at comparable stages of growth the plumule leaves (2 cm. long) have a urease activity per gram of fresh weight of over 15 times that of the ternate leaves of the same size; likewise, the young epicotyl (second internode) has a urease activity per gram of fresh weight of over 20 times that of an upper internode of similar weight. The cause of this difference in urease activity may be traced back to the development of the maturing seed, during which period there is a marked protein synthesis and storage including urease synthesis and storage. Upon germination, the meristematic cells of the radicle and plumule not only start with a high content of urease but are supplied by the cotyledons with a large amount of nitrogenous compounds from which to synthesize more urease during cell division and cell elongation. The later-developing structures are poorer in urease content, which is probably due to the fact that they are less richly supplied with nitrogenous compounds and must depend for their nitrogen on the mature and senescent cells and upon the supply of inorganic nitrogen obtained from the soil.

Summary

1. The urease activity of *Canavalia ensiformis* has been determined using histological and quantitative methods. The data for total urease are summarized in figure 6 which is a graph showing the total urease content of the developing bean, and of the young plant through the 40-day-old stage. The distribution of urease in the various tissues of seedlings 10, 20, 28, and 40

days old is illustrated in a composite picture (figs. 1, 5). Stippling indicates the relative urease concentrations as revealed by the histological methods. The figures opposite each plant part express the urease activity in terms of urease units per gram of fresh weight as determined by the quantitative method. A description of the specific urease changes in the individual plant structures is given under their respective headings in the previous pages.

2. The changes observed in the urease content of the plant are due primarily to the changes in urease content of the parenchyma cells. Neither the cambium nor the cells derived from the cambium, that is, the phloem and xylem, contain any amounts of urease that can be detected with the indicator reagents.

3. The changes of urease activity in the parenchyma cells of any organ follow the same trends with aging of the cell. There is a rapid synthesis of urease in actively dividing cells. This synthesis continues during the elongation stage of the cell, the urease content of a cell reaching a maximum at the end of the elongation stage. After this stage, there is a decrease of the enzyme which continues until the urease content of the cell is down to a certain level.

4. The cells of the embryo developing from the fertilized egg and laid down in the maturing seed possess a urease activity which is higher than that of cells in similar organs developing in the seedling or mature plant.

The writer desires to express his gratitude to Professor F. G. GUSTAFSON for the many helpful suggestions and valuable criticisms throughout the course of this investigation and to Mr. HARMAN DUNHAM for assistance in taking the photomicrographs and in the preparation of the illustrations.

UNIVERSITY OF MICHIGAN
ANN ARBOR, MICHIGAN

LITERATURE CITED

1. DOUTT, M. T. Anatomy of *Phaseolus vulgaris* L. var. Black Valentine. Michigan State Agr. Sta. Tech. Bull. 128. 1932.
2. GRANICK, S. Urease distribution in plants: General Methods. Plant Physiol. **12**: 471-486. 1937.
3. LINDERSTRØM-LANG, K., AND HOLTER, H. Ueber die Peptidaseverteilung in Wurzel und Blattkeim des Malzkornes. Zeitschr. physiol. Chem.
4. PIPER, C. V. The Jack bean. U. S. Dept. Agr. Dept. Circ. 92. 1920.
5. ————. American species of *Canavalia* and *Wenderothia*. U. S. Nat. Herb. **20**: 555-588. 1925.
6. SUMNER, J. B. Crystalline urease. Ergeb. der Enzymf. **1**: 295-301. 1932.

UREASE DISTRIBUTION IN *SOJA MAX*¹

SAM GRANICK²

(WITH FIVE FIGURES)

Introduction

In this account, the distribution of urease, as studied by histological and quantitative methods (10), will be discussed under the sub-headings of the various plant structures.

The activity of the enzyme is expressed in U.U., a urease unit, which is the quantity of urease that will produce 1 mg. of NH_3 per minute when acting upon urea under the conditions employed in the analyses.

Soja max var. Manchu is an upright leafy branching annual legume. The appearance of the plant is much affected by the conditions under which it is grown. In the field, the plants produce a luxuriant growth, forming large leaves, stout, woody stems, numerous pods, and branching is frequent. The plants grown in the greenhouse at Ann Arbor were three feet tall at maturity and rarely branched. The same variety of bean, grown at Woods Hole, Massachusetts, in wooden boxes in soil 6 inches deep, attained a height of only 8 inches and bore 1 to 2 pods per plant. Unless otherwise stated, the determinations described in the following pages were made on plants grown in the greenhouse at Ann Arbor. The reader is referred to the first paper of the series (10) for details as to sampling of material for analysis, etc. The distinctive taxonomic description of *Soja max* var. Manchu is given by ETHERIDGE, HELM, and KING (6).

Investigation

COTYLEDONS OF THE GERMINATING SEED

The seed of the soy bean, because of its commercial importance, has been made the subject of numerous chemical investigations. Since data on the chemistry of the seeds were available, it became interesting to attempt to find a correlation between urease and the various chemical substances present in the seed. As was to be expected from the fact that urease is a globulin, the changes of urease were found to be related directly to the general changes in protein content. The cotyledons of jack bean contain much protein and starch. The cotyledons of soy bean are notable for their high protein and fat content, and at maturity contain less than 0.5 per cent.

¹ This is the third of a series of papers on urease distribution in *Canavalia ensiformis* and *Soja max*. For more extensive data and discussion, the reader is referred to the original thesis available at the University of Michigan library.

² Newcombe Fellow in plant physiology.

Paper no. 612, Department of Botany, University of Michigan.

starch. Both kinds of seeds possess the highest reported urease activity of any materials yet found. SUMNER (24) estimates that soy beans contain about 0.01 per cent. urease by dry weight.

The histological urease reagents showed that urease was highest in content in the germinating cotyledons. As germination proceeded there was a gradual decrease of the enzyme (figs 1, 2). In the 8-day-old seedlings,

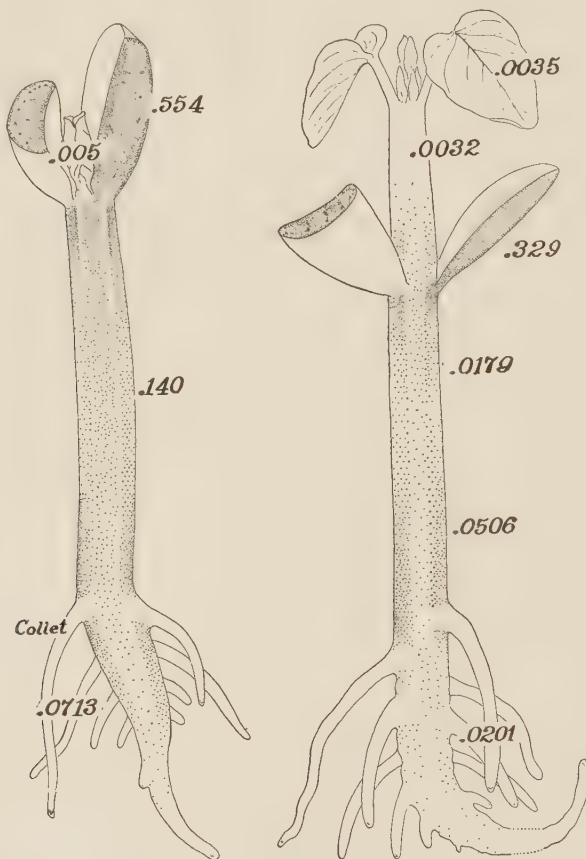


FIG. 1. Composite representation of the urease distribution in the various structures of soy bean seedlings 8 and 15 days old. The number opposite each region of the seedling expresses the number of urease units per gram of fresh weight of that region, as determined by the quantitative method. Stippling indicates the relative urease concentrations as revealed by the histological methods.

the enzyme appeared to be most concentrated at the periphery of the cotyledons, that is, in the subepidermal layers. Urease had diminished markedly in the center of the cotyledons. Some large parenchyma cells gave a stronger reaction for urease than others. The rate of depletion of urease in these

cells showed no evident relation to their proximity to the vascular bundles. The 3 to 4 layers of palisade cells gave a somewhat stronger urease reaction than did the other parenchyma cells. The vascular bundles showed no detectable urease. The content of urease of the bundle sheaths was neither higher nor lower than that of the neighboring parenchyma cells.

Because the chlorophyll content of the outer cells, to some extent, masked the urease reaction, observations were also made on seedlings germinated in

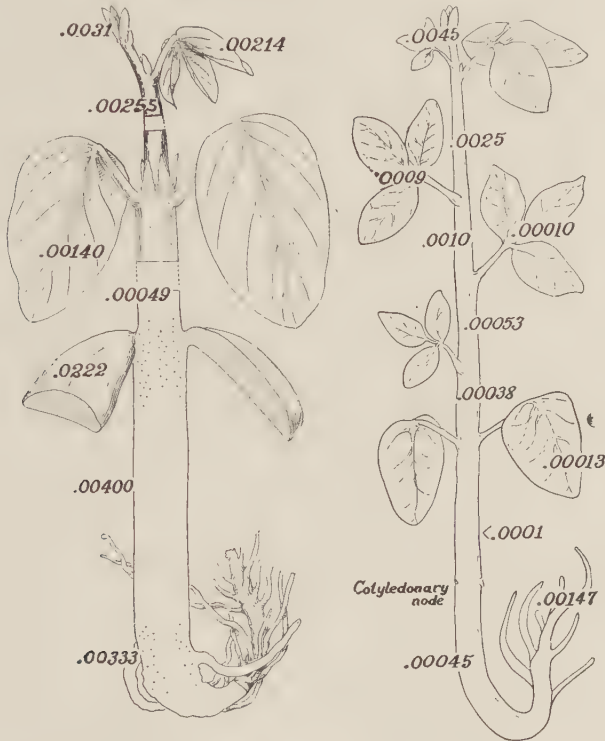


FIG. 2. Composite representation of the urease distribution in the various structures of soy bean seedlings 31 and 52 days old. Numbers have the same meaning as in figure 1.

the dark. There was a more rapid depletion of storage substances from cotyledons of etiolated than non-etiolated seedlings. The urease distribution in the etiolated cotyledons was similar to that in the green cotyledons, except that urease diminished more rapidly in the etiolated cotyledons.

In the 16-day-old seedlings the urease distribution in the cotyledons was similar to that in the 8-day-old plants, except that the total concentration of urease had decreased. The parenchyma cells of the under surface gave a somewhat stronger urease reaction than did the palisade or other parenchyma cells.

In the 30-day-old seedling the cotyledons, which were by now falling off, were yellow, flaccid, and shrivelled. Determinations on these cotyledons generally showed a low urease content.

TABLE I
UREASE CONTENT OF THE COTYLEDONS OF SOY BEAN SEEDLINGS

TEMPERA- TURE DURING GERMINA- TION	DAYS AFTER PLANTING	FRESH WT. IN GM. PER COTYLEDON	DRY WT. IN GM. PER COTYLEDON	U.U. PER GM. FRESH WT.	U.U. PER COTYLEDON
°C.	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
27	0	0.0803	0.0704		
	1	0.1606	0.0693	2.12	0.341
	2	0.1716	0.0688	2.96	0.507
	4	0.1885	0.0554	2.35	0.442
	6	0.306	0.0414	1.48	0.453
20	3	0.176		1.80	0.317
	7	0.215		0.755	0.162
	8	0.222		0.554	0.123
	15	0.356		0.329	0.117
	31	0.263		0.0222	0.00844

A quantitative study was made of the changes in the quantity of urease in the cotyledons at various stages of germination. The changes in urease content taking place at temperatures of 20° and 27° C. during the process of germination and early development of the seedling are shown in table I. These changes are complicated by various factors of which the following are suggested:

1. There will be a decrease of urease as the proteolytic enzymes split the proteins and urease into smaller, more soluble transport products. The urease content will depend then on the activity of the proteolytic enzymes and the rate of transport of their products away from the cotyledons and into the developing tissues.

2. Proteins may be in a storage phase *i.e.*, in aleurone grains, etc. If some urease is present in this state it cannot be detected, for the enzyme must be in a dispersed state in order that urea molecules may come in contact with it (10). Therefore, it is necessary to consider the rate at which dispersal of the stored urease occurs.

3. There is also a possibility that some urease may be synthesized in the cotyledons during germination.

One may consider that during the first days of germination the storage proteins are dispersed and come into solution in the form of soluble proteins at a rate which is more rapid than the rate at which the soluble proteins are

hydrolyzed. The dispersal of proteins is faster at 27° than at 20° C., since water is taken up more rapidly by the soaking seeds at the higher temperature. At 27° C. the urease activity of the cotyledons is greatest after two days germination. The amount of protein being dispersed soon decreases owing to the fact that little storage protein is left; while the dispersed or soluble proteins continue to be hydrolyzed at a rapid rate. Thus a decrease in the amount of soluble protein will take place, appearing as a decrease in urease activity.

RADICLE

The *radicle* in the 2-day-old seedlings, was about 1.3 cm. Cross sections were made through various portions of the radicle and their urease content was determined. Sections through the tip of the radicle showed that the plerome contained a high percentage of urease; the periblem and dermatogen gave only a faint reaction. Sections through the middle of the radicle showed that the epidermis and tetrarch xylem contained no detectable urease; the pith was faintly positive; the cortex was strongly positive. There seems to be a tendency for urease to disappear more rapidly from the pith than from the cortex. In the jack bean there appears to be a tendency for urease to decrease more rapidly in the cortex than in the pith. In general, however, the distribution of urease in the radicle of soy bean is similar to that in the jack bean.

Examination of the collet region of 8-day-old seedlings showed a high urease content in the wide cortex area and relatively little urease in the pith (fig. 1). The endodermis, the cambium, and the phloem and the xylem of primary and secondary origin appeared to contain no urease. (The difficulty in judging the slight urease content of a group of cells, adjacent to

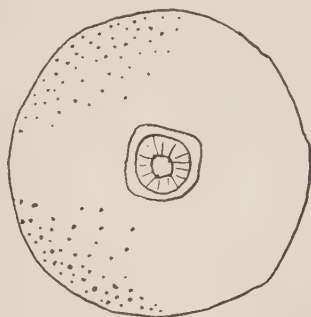


FIG. 3. Urease distribution in a cross section of the collet region of a 39-day-old seedling.

another group of cells containing much urease, has been discussed previously for the collet region of the jack bean.) The bursting of the secondary roots through the cortex causes some of the cortical parenchyma cells at the break to become slightly brownish and to increase in alkalinity to pH 7, or somewhat higher. The young secondary roots were separated from the main root and

tested by themselves. Not more than 50 per cent. of these roots gave positive indications with the urease reagent, and these showed urease to be present at the growing points and nowhere else. Evidently the method is not sensitive enough to detect the low activity of all of these roots. There appeared to be no correlation between the position on the collet axis from which the secondary root arose, and its urease activity at the growing point.

After 16 days urease was still present in the small outer cortical cells. The innermost cortical cells and the stele contained no urease. The collet region loses its urease activity more slowly than any other region. Urease could still be detected in the collet in a 30-day-old seedling but the enzyme was delimited to isolated portions of the outer cortical cells (fig. 3). In the 46-day-old plant no urease could be detected in the collet.

Root

In the 8-day-old seedling the root is about 7 cm. long. Urease was found to be highest in content in the region just below the collet and to decrease rapidly down the root until within 2 cm. of the tip where it could no longer be detected (fig. 1). The outer cortical cells contained the highest amount of urease at the collet region, but as one descended along the root, the urease reaction became diffuse in the cortex. No urease was present in the stele. At the growing point of the root there was a slight amount of urease which decreased so rapidly that it could not be detected in the elongation region. Generally the primary root tips at this stage all gave the urease reaction, in contrast to the secondary root tips which, on the whole, had a lesser urease content and gave indications of urease in less than 50 per cent. of the secondary roots examined. Very little urease was found in the 15-day-old seedling, in the region of the root just below the collet. Urease therefore decreases quite rapidly with age in the roots, except at the growing points.

TABLE II
UREASE CONTENT OF THE ROOT

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
1	Embryo minus cotyledon	0.0122	2.66	0.0324
3	Embryo minus cotyledon	0.0975	0.517	0.0505
4	Root and collet	0.105	0.0973	0.0102
8	Root and collet	0.131	0.0713	0.0093
15	Root and collet	0.274	0.0201	0.0055
31	Root and collet	0.419	0.0033	0.0013
52	Root and collet	1.36	0.0014	0.0020

In table II are shown the changes in urease content that take place in the root with age. There is a rapid decrease of urease activity per gram of

fresh weight; a rise of total urease per structure to a maximum two days after planting and then a decrease of total urease after that day. Even though the roots have increased rapidly both in weight and number of secondary roots, the total urease content decreases. As will be shown later, this rapid decrease in urease content may be related to the fact that the elongation region of the root is a relatively short one, and the elongating cells mature rapidly.

HYPOCOTYL

In the 8-day-old seedling urease had already begun to decrease sufficiently so that differences in concentration could readily be noted in the hypocotyl with the histological urease reagent (fig. 1). The enzyme content was greatest at the cotyledonary node and at the collet. Urease was found only in the parenchyma cells of cortex and pith; never in the cambium or its derivatives. The urease content of the pericyclic cells could not be determined with certainty, although there are indications that these cells do contain urease. Sections through the cotyledonary node showed the pith to contain slightly less urease than the cortex. The enzyme decreased rapidly in the pith from the upper to the lower part of the hypocotyl. At the top of the hypocotyl there appeared to be more urease in the cortical parenchyma cells adjacent to the stele. There was a gradual decrease in urease content in the cortex from the top of the hypocotyl to the lower end, but, on nearing the collet, urease appeared to become more concentrated in the outer smaller cortical cells.

In the 16-day-old seedlings the differences in enzyme content of the various cells had become more marked. The hypocotyl, as seen in cross section just below the cotyledons, still gave strong urease reactions. Owing to elongation there was a mechanical thinning out of the enzyme at this stage, taking place most rapidly in the upper two-thirds of the hypocotyl. In the remainder of the hypocotyl urease became restricted to the outer cortical cells and little of the enzyme was to be found in the pith.

In the 30-day-old seedlings (fig. 2) urease was detected only at the cotyledonary node, and just within the collet region; in both these instances urease was restricted to the outer cortical parenchyma cells. In the 50-day-old plants (fig. 2) no urease could be detected in any portion of the hypocotyl.

Within a period of seven days the hypocotyl had increased from 3 to 60 mm. It will be noted from the data of table III that after the eighth day of germination primary growth and elongation of the hypocotyl had practically ceased. Even before this time the maximum total urease content of the hypocotyl had been attained. The phases of primary growth and maturity overlap. The quantitative data of table III and the results obtained with the histological reagents showed that urease was not only being me-

TABLE III
UREASE CONTENT OF THE HYPOCOTYL

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
1	Embryo minus cotyledon	0.0122	2.66	0.0324
2	Embryo minus cotyledon	0.0975	0.517	0.0505
8	Hypocotyl 6 cm.	0.233	0.140	0.0326
15	Hypocotyl 7 cm.	0.404	0.0347	0.0140
	Upper half	0.194	0.0179	0.00347
	Lower half	0.210	0.0506	0.0106
31	Hypocotyl	0.434	0.00400	0.00173
52	Hypocotyl	0.500	0.00045	0.00020

chanically thinned out, but that already in the late stages of primary growth the enzyme was actually being inactivated or catabolized so that the total urease content of the hypocotyl decreased with further age.

STEM

The apical meristems possess a urease activity that is too low to be studied with the histological reagent. From quantitative data there is reason for believing that there is no difference in urease distribution between the apical meristems of the soy bean and the jack bean, except the difference in urease concentration.

The epicotyl or second internode³ is considered apart from the other stem internodes because of its proximity to the cotyledonary node. In the 9-day-old seedling the epicotyl is about 5 mm. long. Cross sections through the tip of the epicotyl, where the first leaves arise, showed urease to be present in the outer cortical parenchyma cells and also faintly in the pith.

In the 16-day-old plant (fig. 1) the epicotyl showed the presence of urease only in the region close to the cotyledonary node; the urease reaction had diminished considerably in intensity. No cells except the parenchyma cells of the cortex, and more faintly the pith, gave a positive test with the urease reagent. In the 30-day-old plant no urease could be detected in the epicotyl.

The quantitative data for the urease content of the epicotyl are given in the table IV. The figures show that the most rapid elongation occurred between the eighth and fifteenth day, during which time the total urease per

³ For convenience the hypocotyl has been designated as the first internode although this is not technically correct according to the terminology of the morphologist. The epicotyl is considered as the second internode and the other internodes are numbered successively upwards.

structure increased markedly, while the urease activity per gram of fresh weight decreased slightly. Although elongation still occurred after the fifteenth day, it was relatively slight. The phases of primary and secondary growth overlap each other. It may be concluded that the total urease content is at a maximum during the period of the most rapid elongation of the cells of primary growth and thenceforth urease begins to decrease in activity.

TABLE IV
UREASE CONTENT OF THE EPICOTYL (SECOND INTERNODE)

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
8	5 mm. plumule	0.0116	0.005	0.00005
15	2nd internode 6 cm. ...	0.118	0.0032	0.00030
31	2nd internode 9 cm. ...	0.325	0.00049	0.00015
52	2nd internode 10 cm.	0.509	0.0001	0.00005

Examination of sections made of the other internodes, above the second, and tested with the urease reagents indicated that the amount of urease present even in the youngest internodes was too small to be detected.

Quantitative determinations were made of the urease content of the internodes above the second and the data are shown in table V. Although growth in length is not exactly the same for each internode, (the weight of the sixth internode, for example, being greater than the weight of any older internode) the changes of urease with age are still sufficiently marked to be

TABLE V
UREASE CONTENT OF INTERNODES OF A 52-DAY-OLD PLANT

STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	TOTAL U.U. PER STRUCTURE
	<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
7th internode including leaflets and apical bud	0.064	0.0031	0.00018
6th internode	0.250	0.0025	0.00062
5th internode	0.223	0.0010	0.00020
4th internode	0.171	0.00053	0.00009
3rd internode	0.241	0.00038	0.00008

clearly recognized. These changes in urease content are similar to those undergone by other plant structures. The urease activity steadily decreases with age, the oldest internodes having the lowest urease activity per gram of fresh weight. The total urease per internode is highest in the sixth

internode. This internode has just finished its maximum primary growth. From that time, the total urease content diminishes with the maturation and senescence of the internode.

LEAVES

In the 1-mm. first foliage or plumule leaves of the ungerminated soaked seed urease was found in all the cells except the primary xylem. The histological reagent also showed that urease decreased rapidly as the leaf expanded.

The data on the urease content of the first pair of foliage leaves of various ages are presented in table VI. The figures in the table indicate the same trend for changes in urease content as was found in the structures previously examined *i.e.*, a decrease of urease activity per gram of fresh weight; and for the total urease content per structure, at first a rise to a maximum which coincides with the period of maximum elongation, then a slow decrease after primary growth has ceased.

TABLE VI
UREASE CONTENT OF THE FIRST PAIR OF FOLIAGE LEAVES

DAYS AFTER PLANTING	STRUCTURES	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
8	Plumule—5 mm.	0.0116	0.005	0.00005
15	Leaves—3 cm. and petioles—4 mm.	0.170	0.0035	0.00060
31	Leaves—4.5 cm. and petioles—1.5 cm.	0.387	0.00140	0.00053
52	Leaves—5 cm.	0.405	0.00013	0.00005

Sections through the embryonic compound leaves in the apical buds above the second node gave no indication of the presence of urease when the histological method was used. The urease content in the meristematic tissues of the stem, and in the leaf primordia is too low to be detected by this method.

Determinations were made on the trifoliate leaves of soy bean plants grown in the field during the summer, and on others grown in the greenhouse during the winter. The plants grown in the field were harvested at the stage when they possessed pods that were almost mature. The quantitative data (table VII) show the same general trend of urease metabolism as do the plumule leaves. The leaves of the field-grown plants were larger, weighed more, and possessed a urease activity which was maintained at a high level even after primary growth had ceased. The maximum size of the field-grown leaflet was 11 cm. as compared to 6 cm., the maximum size of the

TABLE VII
UREASE CONTENT OF COMPOUND LEAVES

LEAF		FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER LEAF
		<i>gm.</i>	<i>U.U. gm.</i>	<i>U.U.</i>
Greenhouse-grown plants 52 days old				
7th node	3 cm. leaflets	0.355	0.0045	0.0015
6th node	6 cm. leaflet	0.777	0.0013	0.0010
	7 cm. petiole			
5th node	6 cm. leaflet	0.673	0.0009	0.00060
	7 cm. petiole			
4th node	5 cm. leaflet	0.463	0.0001	0.00004
	7 cm. petiole			
Field-grown plants with nearly mature pods				
16th node	4 cm. leaflet	0.414	0.00517	0.00214
	2 cm. petiole			
15th node	7 cm. leaflet	1.03	0.00467	0.00454
	5 cm. petiole			
14th node	9 cm. leaflet	2.28	0.00409	0.00932
	8 cm. petiole			
13th node	11 cm. leaflet	3.31	0.00346	0.0114
	10 cm. petiole			
12th node	11 cm. leaflet	3.11	0.00264	0.00821
	10 cm. petiole			
11th node	11 cm. leaflet	3.98	0.00238	0.00946
	13 cm. petiole			
10th node	11 cm. leaflet	3.16	0.00227	0.00718
	13 cm. petiole			
9th node	9 cm. leaflet	2.34	0.00294	0.00688
	8 cm. petiole			
8th node	8 cm. leaflet	2.09	0.00258	0.00539
	9 cm. petiole			

greenhouse-grown leaflet, and the weights of each were respectively 3.98 and 0.777 gm. The leaves of the plants grown in the field had a urease activity of 0.005 to 0.002 U.U. per gram of fresh weight, whereas the urease activity of leaves from greenhouse-grown plants was 0.004 to 0.0001 U.U. per gram of fresh weight. If it is assumed that urease activity follows changes in soluble protein content, then it might be said that the greenhouse-grown leaves behaved as if there was a lack of available carbohydrate in the plant for maintaining a high level of protein nitrogen, the depletion of urease and protein taking place rapidly when the leaves had finished expanding because of the drain of carbohydrate and non-protein nitrogen from them into the growing points. However, even under the different environmental conditions of summer and winter growth, expressing themselves in such a wide divergence of growth in green weight and in urease content, the same general scheme of urease metabolism in the leaves was observed as had been noted for the other structures of the plant, namely, that the highest urease

activity was present in the youngest leaves and decreased with age of the leaf; and the total urease content increased during primary growth and then decreased.

FLOWER AND FRUIT

The urease content of the flower primordia, the flower, and the pod tissues is below the concentration that is detectable with the histological reagents. The quantitative changes of urease content in the developing pods may be noted from the data in table VIII. The course of the change in urease activity

TABLE VIII
UREASE CONTENT OF POD

STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
	<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
Young flowers and buds		0.006	
Pod (contained 3 mm. seeds)	0.1490	0.0051	0.00076
Pod (contained 5 mm. seeds)	0.2010	0.0042	0.00084
Pod (contained 9 mm. seeds)	0.2518	0.0025	0.00063
Pod (contained 11 mm. seeds)	0.371	0.0011	0.0004

is similar to that found for other tissues, that is, there is a steady decrease of urease activity per gram of fresh weight; and an increase and then a decrease in the total content of urease per pod.

THE PLANT AS A UNIT

A survey of the changes in the total urease content of the developing seeds and of the seedlings of soy bean is given in a graph (fig. 4). Along the abscissa the relative age of the bean or plant is represented. On the ordinate is plotted logarithmically the total urease values. It may be observed from this graph that the urease content increased almost ten thousand-fold from the youngest seed examined (3.5 mm. long) to the mature seed (13.5 mm. long). Although in the last stages of maturity the seeds were beginning to dry, decreasing in length from 13.5 to 12 mm., and in water content from 63.3 to 48.6 per cent., the urease content still increased. Finally, at the last stage, when the seed had shrunk to 9 mm. and the water content decreased to 19.4 per cent., the urease content had decreased to approximately one-sixth the value of the previous stage.

The completely mature and dry seeds were then germinated. Those planted at 27° C. showed an increase in urease content which reached a maximum at the second day and then began to decrease. Plants grown at 20° C. had a lower level of total urease per plant. There was a rapid decrease of total urease per plant from the fifteenth day.

As with the jack bean, the curves illustrating the changes in total urease content per seed or per plant really indicate the changes of urease taking place in the cotyledons, since these structures contain most of the enzyme. In the developing seed, the cotyledons, especially in the later stages, consti-

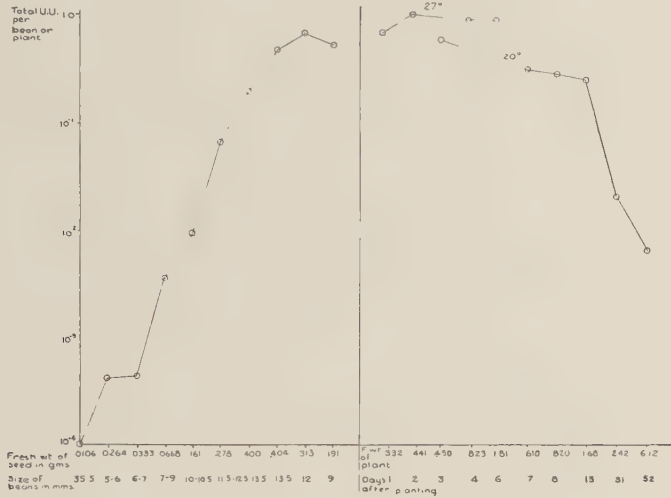


FIG. 4. The graph represents the total urease content of the developing beans, or of the seedlings of soy bean, at different stages of growth. Along the ordinate, total U.U. values are plotted logarithmically; along the abscissa, relative ages of the beans or seedlings are represented.

tute over 90 per cent. of the dry weight of the seeds. As the seeds increase in size, urease increases very rapidly. In the last stages when the seeds begin to dry (as indicated by the pods turning yellow, the hila of the seeds changing from green to pink then to purple and finally to black, and the cotyledons from a green to a yellow-green and finally to yellow) urease increases and finally decreases. This decrease in urease activity is interpreted as a storage of the enzyme in a form which is not readily dispersed in aqueous solution.

In the 31-day-old plant, the cotyledons, which are about to fall off, still contain approximately 80 per cent. of the total urease of the plant. The marked decrease in urease content between the 15-day-old and 31-day-old plant is due primarily to the rapid decrease in urease of the cotyledons themselves. Even after the cotyledons have fallen off, however, the total urease of the plant continues to decrease.

A composite picture of the distribution of urease in the various parts of the developing seedlings of soy bean 8, 15, 31, and 52 days after planting is represented in figures 1 and 2. The stippling indicates the relative urease content as revealed by the histological methods. The numbers opposite each

plant part are the number of urease units per gram of fresh weight of that plant part as determined by the quantitative method.

Discussion

COMPARISON OF UREASE DISTRIBUTION IN SOY BEAN AND JACK BEAN

It was shown in the second paper of this series (11) that, in all the organs and plant structures examined, the urease changes follow the same course. The urease activity of an organ per gram of fresh weight (curve A, fig. 5)

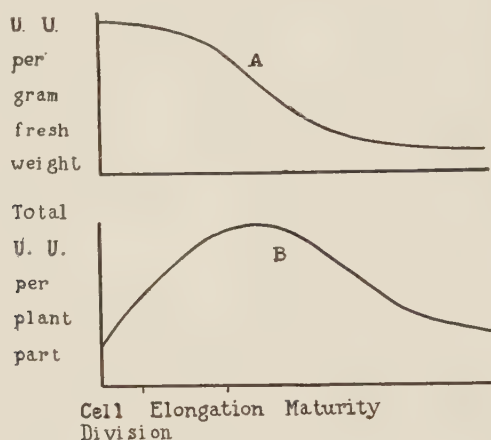


FIG. 5. Changes of urease activity in relation to physiological age. Curve A, change of urease activity, of an organ per gram of fresh weight, with age; curve B, change of total urease content of an organ with age.

is highest when the cells are still meristematic and decreases as the cells grow older. The total urease content of an organ (curve B, fig. 5) increases rapidly during the period of cell division until, at about the time primary growth has ceased, the organ contains a maximum urease content. From that time on, there is a decrease in urease content which is more or less marked, depending on the individual organ considered. It was further shown that the changes in urease activity noted in the organs are due primarily to changes taking place in the parenchyma cells of the organs since neither the cambium nor its derivatives contain detectable amounts of the enzyme. The total urease activity of the meristematic cell increases rapidly at first (curve B, fig. 5); and as the cell passes into the stage of elongation the rate of increase of urease activity drops off. About the time the cell has acquired its maximum size it contains a maximum amount of urease. After this time there is a gradual decline of urease activity per cell until some constant low level is reached. This series of changes found in *Canavalia ensiformis* is likewise found in *Soja max*.

There were, however, some minor differences. In general the urease concentration was less in soy bean than in jack bean. The palisade cells of the soy bean cotyledon contained somewhat more urease than did the other parenchyma cells; and in the 8-day-old seedling, urease was present in somewhat greater concentration at the periphery of the soy bean cotyledons than toward the inner region. In the jack bean cotyledon no such differences in distribution were observed. There was a more rapid decrease in urease activity in the pith than in the cortex of the soy bean hypocotyl during germination. In the jack bean, however, the decrease in the hypocotyl was somewhat more rapid in the cortex than in the pith.

UREASE IN OTHER LEGUMES

That the curve of urease changes is not merely limited to the two plants investigated but holds also for other legumes is indicated by the experiments of KIESEL and TROITZKI (12). Their limited data on the urease changes in a number of legume plants are in accord with the general changes of urease in the jack bean and soy bean plants. For example, the young developing seeds of *Phaseolus vulgaris* and *Pisum sativum* contained one-fifth the urease per gram of fresh weight of the older seeds. The leaves of a *Pisum* seedling three weeks old contained more urease per gram of fresh weight than did the stems. Cotyledons of 10-day-old seedlings of *Vicia faba* had about five times the urease activity per gram fresh weight that cotyledons of 28-day-old seedlings had. The changes in urease distribution and concentration found in the soy bean and jack bean may then be considered as changes which are at least common to the Papilionaceae.

A COMPARISON OF DISTRIBUTION OF UREASE AND OTHER ENZYMES

BURK (5) has called attention to enzymic activities which are growth-bound, *i.e.*, "where the enzymic activity is correlated with the structure of the living cell to the extent that the velocity of formation of the (intracellular) reaction product parallels and is normally measured by the velocity of growth. Such growth-bound enzymes may be termed phyto-enzymes."

Since the enzyme urease appears to be so intimately bound up with the growth of the parenchyma cell, one may consider urease as a growth-bound enzyme. It was interesting to see whether other hydrolytic enzymes might likewise be growth-bound and undergo changes similar to those found for urease. In table IX data are presented on the analyses of urease, protease, and lipase in the developing seed. The urease content increases markedly during the period of seed ripening. TOVARNITZKY'S analyses (25) of the nitrogenous fractions of soy beans at different stages of development show that the increase of urease activity parallels the increase in the soluble protein content and this investigator calls attention to the fact that "A reliable

TABLE IX

ANALYSES OF SOY BEAN SEEDS AT DIFFERENT STAGES OF DEVELOPMENT

ANALYSES	SIZE OF BEANS IN MM.							
	3.5-5	5-6	6-7	7-9	10-10.5	11.5-12.5	13.5*	13.5†
							12 YELLOW BEANS DRYING	9 YELLOW BEANS DRY
Amino-N in mg.								
Per gm. fresh wt.	2.94	2.84	2.88	2.92	2.34	2.16		5.72
Per gm. dry wt.	12.0	17.1	18.9	13.4	9.87	6.85		7.10
Per bean	0.0311	0.0750	0.0960	0.195	0.376	0.600		1.09
Free NH ₃ in mg.								
Per gm. fresh wt.	0.156	0.145	0.156	0.124	0.130	0.184	0.192	0.124
Per gm. dry wt.	0.588	0.871	1.02	0.568	0.549	0.583	0.522	0.154
Per bean	0.00165	0.00382	0.00519	0.00282	0.0209	0.0512	0.0766	0.0239
Urease activity in mg. NH ₃ produced per min.								
U.U. per gm. fresh wt.	0.010	0.0164	0.0139	0.0555	0.0596	0.243	0.497	2.92
U.U. per gm. dry wt.	0.037	0.0986	0.0891	0.254	0.252	0.770	1.35	3.65
U.U. per bean	0.00010	0.00043	0.00048	0.00370	0.00958	0.0675	0.199	0.558
Protease activity in mg. NH ₃ -N produced in 48 hr.								
Per gm. fresh wt.	12.85	11.00	11.85	10.60	10.00	7.30		9.40
Per gm. dry wt.	48.4	66.1	77.7	48.6	42.2	23.2		11.7
Per bean	0.136	0.290	0.395	0.708	1.61	2.03		1.80
Lipase activity in cc. of 0.1 N acid produced in 48 hr.								
Per gm. fresh wt.	3.36		3.40	2.74	3.86	4.68		1.12
Per gm. dry wt.	12.6		22.3	12.6	16.3	14.8		1.39
Per bean	0.0356		0.113	0.183	0.621	1.30		0.214
Titration acidity in cc. of 0.1 N acid								
Per gm. fresh wt.	0.432		0.381	0.341	0.352	0.203		0.284
Fresh wt. per bean	0.0106	0.0264	0.0333	0.0668	0.161	0.278	0.400	0.191
Dry wt. per bean	0.00281	0.00439	0.00507	0.0146	0.0382	0.0876	0.147	0.154
Percentage water in bean	73.5	83.3	84.8	78.1	76.2	68.7	63.3	48.6

* Hilum light pink; pod turning yellow.

† Hilum dark purple; pods dark yellow; beans yellow green.

index for the amount of protein in soy beans, its solubility and the maturity of the beans, is the activity of urease." As the bean matures, urease accumulates in the cells, some of it being stored in the form of ergastic amorphous substances just as other globulins are stored. The water content in the later stages is decreasing, the cellular materials becoming more and more concentrated; and the semi-fluid vacuoles of the cells take on the appearance of solid spherical corpuscles. Although the storage phase is evident at the last stage (*i.e.*, a decrease of urease activity in the 9 mm. yellow bean) it is highly probable that some storage of the protein urease has occurred even in the earlier stages of seed development.

This increase of urease activity as the seed matures is not paralleled by an increase of protease or lipase activity.⁴ It is seen from table IX that the protease and lipase activities per gram of dry weight of seed increase at first, reach a maximum activity in the 6- to 7-mm. bean and then decrease as the bean enlarges. It is interesting to note that the values for amino nitrogen and free ammonia per gram dry weight, and for the percentage of water content are also highest in the 6- to 7-mm. bean. These results on protease and lipase activities of the developing bean do not agree with those of RIVKIND and TOVARNITZKY (25) who report a slight increase of these enzymes in the maturing seed. These investigators made analyses on the air-dried seeds, and do not describe their methods of analyses. The determinations reported in table IX were made on freshly picked material without preliminary drying; corrections were made for the varying values of autolysis, which become important in estimating the very low activities of both protease and lipase found in the soy bean. In this connection it may be mentioned that, in the developing seeds of *Vicia faba*, BLAGOWESTSCHENSKI (3) found that the amylase content per gram dry weight of seed decreased as the seeds ripened.

Upon germination of the seeds the aleurone grains and crystalloids absorb water and are seen gradually to take on a semi-fluid appearance. In the beans grown at 27° C. (table I) the values for urease activity per gram of fresh weight of the cotyledon increased up to a maximum value on the second day and then began to decrease. [The germinated beans, used for the determinations on protease and lipase (table X), were not those related to the developing beans of table IX, so that no direct comparison can be made between the protease and lipase activities of the developing and germinating beans.]

⁴ Proteinase activity was measured by permitting aliquots of fresh tissue mash to act on a 2 per cent. casein solution for 48 hours at 30° C. in the presence of phosphate buffer (pH 6.5) and overlaid with toluene. Amino-N was determined by the Van Slyke nitrous acid method. Lipase activity was measured by the increase in titratable acidity of a gum arabic-castor oil emulsion, containing about 2 per cent. fat, when acted upon by 0.5 per cent. tissue mash for 48 hours at 30° C. and overlaid with toluene. (Toluene does not interfere with the determination.) Titration to the phenolphthalein end-point is made on an aliquot of an alcohol-ether extract of this mixture.

The protease activity per gram of fresh weight of the cotyledon reached a maximum in two days and then slowly decreased. From the complete hydrolysis of proteins there will be produced some 7 mg. $\text{NH}_2\text{-N}$ per bean. This indicates that protein digestion during germination is significantly limited by the low protease activity. Lipase activity increased in two days and was apparently highest in the 11-day-old stage; it then rapidly decreased. In a microchemical examination of the germinating soy bean

TABLE X
ANALYSES OF COTYLEDONS OF GERMINATING SOY BEANS

	DAYS AFTER PLANTING						
	1	2	5	8	11	15	30
Fresh wt. per cotyledon pair	0.274	0.286	0.590	0.512	0.496		0.264
$\text{NH}_2\text{-N}$ in mg. per gm. fresh wt.	2.32	2.70	2.98	3.40	1.68	0.861	
Protease activity in mg. $\text{NH}_2\text{-N}$ produced in 48 hr. per gm. fresh wt.	3.30	4.25		2.70	1.45	0.27	0.078
Lipase activity in cc. of 0.1 N acid produced in 48 hr. per gm. fresh wt.	1.48	3.41	2.24	2.39	4.58	0.86	0.00

VON OHLEN (26) reports that in the cotyledons a marked decrease of oil had occurred by the fourth day; depletion began at the base of the cotyledons and progressed toward the opposite end; the palisade layer was depleted last. The acidity produced by the complete hydrolysis of the fat per bean is equivalent to 1 cc. of 0.1 N acid. The small amount of lipase present and the fact that the fat decreased markedly by the fourth day indicate that the rate of fat hydrolysis was limited greatly by the amount of active lipase present. On germination urease and protease increase during the first two days and then decrease, while lipase attains a maximum activity on the eleventh day and then decreases rapidly.

The data of FISCHER (9) indicate that the proteases of peas, beans, and buckwheat increase in the leaves with age. This differs from the changes of urease found in the leaves examined by the writer in which there is a rapid increase in urease to maturity and then a decrease. In the root tip urease is highest in concentration in the region of cell division and decreases rapidly in the elongation region. Peptidase of the barley root tip, however, is highest (18) in concentration in the region of cell elongation and not in the region of cell division.

UREASE AND RESPIRATION

If urease, unlike protease and lipase, is a growth-bound enzyme it might be expected that the changes in respiratory activity would normally parallel

the changes in urease activity, and that both of these factors would parallel, and be a measure of growth changes. A comparison of the data on the respiratory activity of legumes, as found in the literature, with the data on urease activity of parenchymatous tissue as here presented confirmed the suggestion that urease and respiratory activity run parallel. BEZAGU (2) working with leaves of various ages, of such species as *Robinia pseudoacacia*, *Cercis siliquastrum*, *Pinus sylvestris*, *Cobaea scandens*, *Ligustrum vulgare*, *Althaea*, and *Lovoglossum hercinum* found that the following facts were general for the respiratory activity of these leaves.

1. The amount of CO_2 produced per gram of fresh weight per hour is highest in the youngest leaves and continues to decrease with the development and aging of the leaves in a manner similar to curve A, figure 5.

2. The CO_2 production per leaf-hour is lowest for the very young leaves, increases rapidly to a maximum during elongation, and then gradually decreases as the leaf passes through the stages of maturity and senescence. These changes may be represented by a curve similar to B, figure 5.

This relation of respiratory activity to urease activity, that is, to the fact that urease is a growth-bound enzyme, is understandable upon the basis of the following assumptions:

1. Urease, in its active state, is a soluble protein which undergoes changes similar to other proteins in synthesis and decomposition.

2. The amount of soluble proteins (as distinguished from ergastic proteins), including urease, is a measure of the amount and activity of protoplasm.

3. Respiratory activity is related to protoplasmic activity or to the amount of catalytic (protein) surface upon which oxidation-reduction reactions take place.

UREASE AND PROTOPLASM

Much of the framework of protoplasm (23) is considered to be made up of proteins which are dispersed in an aqueous medium. The only measure of protoplasmic activity which is generally recognized is respiratory activity. Since urease forms a portion of the protoplasmic framework, it is suggested that the amount of protoplasmic framework will be proportional to the amount of active urease and to the respiratory activity. If these assumptions are correct, then the changes in protoplasmic framework or protoplasmic activity in a cell may be studied by observing the changes in urease activity under varying environmental conditions. In other words, the enzyme urease may be used as a "tracer." Although changes in respiration can be much more readily followed than urease changes in the study of protoplasmic activity, they have the disadvantage of being much more complex summation effects of numerous enzymic actions and various substrate concentrations than are the urease changes.

In the young embryonic cell there is a dense protoplasm which, as the cell elongates, later contains a number of vacuoles. With increasing age of the cell, the vacuoles combine to form a single large sap cavity so that the protoplasm becomes spread in a thin film along the cell wall. The data on respiratory and urease activity of leaf parenchyma cells are in accord, not with the interpretation that the protoplasm of the embryonic cell is merely stretched and diluted but that the total amount of protoplasm, or the protoplasmic activity per cell, actually continues to increase until the cell has attained its maximum size; after that, it decreases to a certain level as the cell ages.

UREASE AND PHYSIOLOGICAL AGE

As was noted in the first paper of this series (10), it is only the enzyme in a dispersed state, in which its active groups are available, which can be quantitatively determined. An interpretation of the curve of urease changes with age of the cell is therefore difficult to make. It seems certain, however, that the ascending portion of curve B figure 5, illustrating the increase of urease as the cell enlarges, can be interpreted in no other way than as a synthesis of the enzyme. It has been suggested as a possibility that an enzyme might not be synthesized *in situ* but might be transported as such to the growing regions. For example, the 10,000-fold increase of urease content from the youngest to the oldest seed of soy bean might be due to a transport of urease to the developing seed. The studies on urease indicate that the enzyme is not transported as such to other cells, for the following reasons: (1) Urease has not been detected in the secondary phloem tissue. (2) The disappearance of urease from the storage parenchyma cells of a cotyledon bears no observable relation to the proximity of these cells to fibrovascular bundles in their neighborhood. (3) When jack bean cotyledons, which contain the highest recorded amounts of urease, are kept in tap or distilled water for several days, the water will give no test for the presence of urease. These results may be due to the fact that this enzyme, being a protein molecule, cannot diffuse to any extent through the plasma membrane. It is interesting to note, in this connection, BACH's report (1), that urease is present in *Aspergillus niger* as an endocellular enzyme and that no urease is excreted into the culture medium.

A satisfactory interpretation of the descending portion of curve B figure 5 must await a better understanding of the processes leading to senescence and death of a cell. It is difficult to evaluate the significance of the decrease in urease activity after the cell has reached the end of primary growth since the active enzyme may disappear because it is being catabolized by proteolytic ferments, or because it is being stored in a zymogen form, or because it is being inactivated. There is some evidence that catabolism of urease is

the major cause of the descending portion of the urease curve. From the work of SCHULZE and SCHÜTZ (22) and others it is known that leaves increase in protein N as they enlarge, and that during midsummer the content of protein N per leaf then begins to decline. This decrease in protein and in urease in the mature and aging leaves may perhaps be related to the fact that with increasing age of the leaf there is an increase in protease activity in the leaf (9). Peculiarly enough, the changes in urease and protease of the higher plants appear to run parallel with those found for *Aspergillus niger*. BACH (1) found an increase of urease in *Aspergillus*, the urease content reaching a maximum during the first 5 to 7 days and then rapidly declining to about one-sixth of the maximum value by the fifteenth day. This decline in urease content likewise coincided with an increase in proteolytic activity of the fungus. It is hoped in a future paper to deal with the question of the "element constant of protein," as related to the age and carbohydrate content of the cell, and its relation to urease activity.

RÔLE OF UREASE IN INTERMEDIARY METABOLISM

Since urease appears to be present in almost all plants and certainly to be present in all the organs of the jack bean and soy bean, what rôle does this enzyme play in the metabolism of plants? As is well known, an enzyme catalyzes the attainment of the equilibrium of a chemical reaction (4). Whether and to what extent hydrolysis of urea will take place will depend on the equilibrium constant of the reaction and on the presence of urea and water. The synthesis of urea will likewise depend on the equilibrium constant of the reaction and on the presence of ammonium carbamate.

It has been firmly established by MACK and VILLARS (19) and others that urease catalyzes the following reaction:



The forward reaction, that is, the hydrolysis of urea to ammonium carbamate proceeds very rapidly. It can readily be calculated⁵ that about 2000 urea molecules make effective collisions per second with one urease molecule. The decrease in free energy of this hydrolysis is sufficiently great that urea is almost completely transformed into ammonium carbamate.

That urea can be absorbed by a legume plant from a sterile culture medium and can be split very rapidly into ammonia by the urease present in the cells has been reported by KLEIN (13). He has also shown that if large amounts of urea are fed, the ammonia produced becomes so great that signs of ammonia poisoning appear and the plant turns brown and dies. If one stops the feeding of urea, the total urea taken up disappears in a very short time.

⁵ Assuming a molecular weight of 150,000 for crystalline urease; an activity of 26,000 U.U. per gram of crystalline urease (24); one active spot per urease molecule; excess of substrate; and that the reverse reaction is negligible.

The question of the presence in the plant of the substrate, urea, may be considered briefly. The investigations of KLEIN and TAUBÖCK (14) indicate that little free urea is present in the higher plants. The "urea" reported by previous workers was a complex of ureides which are apparently widely distributed throughout the plant kingdom. The ureides of both the higher plants and the fungi are split by boiling with dilute acetic acid; boiling with dilute alcohol does not decompose them. The ureides of higher plants are readily soluble in alcohol; the ureides of fungi are not. They report only two ureides in higher plants: the formaldehyde compound, which is found only in green assimilating plants; and the acetaldehyde compound which is formed in green unlighted or in chlorophyll-free organs as a result of respiratory processes. FOSSE believes that urea is present in combination with glyoxylic acid as allantoic acid. These stable ureide bodies remain in the organs for some time but at certain definite periods are attacked and disappear. Since the enzyme urease is present in the plant, it may be concluded that any free urea, released from the ureide combinations, from arginine, canavanine, purines, guanidine, etc., will be rapidly decomposed to liberate ammonia. The presence of large quantities of urease in the meristematic regions of the jack bean and soy bean suggests that urease performs a "necessary" function in releasing ammonia for protein synthesis.

Concerning the synthesis of urea by urease it has been postulated by ONSLOW (21), EULER (7), and others, that urea might be formed by the action of urease on ammonium carbamate. It has been realized, however, that when urea is brought into contact with urease an almost complete conversion of urea to ammonia results. But there remained the possibility that synthesis could occur in the cell if the cell had some means of removing from the field of enzyme action the small amounts of urea formed. For example, the trace of urea might be used to form a ureide, and then more urea would be produced because of the equilibrium involved.

It is possible to arrive at some definite conclusions as to the significance of urease in the synthesis of urea by making use of the data of LEWIS and BURROWS (16) on the hydrolysis of urea, and on the conversion of ammonium cyanate to urea (17). If urea, ammonium cyanate, ammonium carbamate, ammonium carbonate, or their ionic products are brought into water, an equilibrium will finally be established in which all of these substances will be present in certain definite proportions. For example, it may be calculated, that starting with a 1 M. urea solution in a closed container at 25° C. the products at equilibrium will be approximately 0.998 M. ammonium carbamate-carbonate, 0.002 M. urea, and a trace of cyanate.

Likewise, the maximum concentration of urea that may be synthesized by urease may be calculated. The highest amount of ammonia nitrogen in the jack bean, equivalent to 0.1 M. NH_3 , was found in the radicle of a

16-day-old seedling (14). Assuming that sufficient CO_2 is present to combine with all the ammonia present, then at this ammonia concentration approximately 80 per cent. of the ammonia will be in the form of ammonium carbonate and the other 20 per cent. will be in the form of ammonium carbamate, in a slightly alkaline medium (8). If this plant tissue is assumed to be neutral, the maximum theoretical amount of urea at equilibrium with this concentration of ammonium carbamate will be 1.8×10^{-6} gm. per gram of tissue. Even though the urea thus formed is slight, it might be removed from the sphere of urease action possibly to form a ureide and thus permit further urea synthesis.

Ammonium carbamate is, however, unstable on the acid side of neutrality and is very rapidly converted to carbonate. No ammonium carbamate is formed from ammonium carbonate in plants because plant tissues have a hydrogen-ion concentration that is generally on the acid side of neutrality. Since the enzyme urease catalyzes the reaction of ammonium carbamate to urea and water, no urea could be synthesized because no ammonium carbamate would be present; and no ureides would be formed. It may therefore be concluded that urease in the plant does not function in the synthesis of urea.

From the data on free energy change it becomes evident that no matter what steps are postulated for the synthesis of urea from ammonia and carbonic acid as they exist in the plant, some energy source, such as a coupled oxidation, is required (15). It is interesting to note that asparagine, another storage molecule for ammonia, is, like urea, an amide and also requires a coupled oxidation process to supply the energy for its synthesis (20). Whether urea formation by synthesis from ammonia is to be considered an important factor in explaining the significant quantities of ureides present in plants remains to be seen. The investigations of FOSSE suggested to KREBS (15) that "urea formation in plants follows mainly the same path as urea formation from purines in amphibians" that is, through the hydrolytic formation of urea from ureides rather than through a direct synthesis from ammonia.

The interesting paper by FEARON⁶ ought to be mentioned. FEARON advances the hypothesis that cyanic acid is in some way responsible for urea formation. He believes that carbamic acid may form cyanic acid which together with ammonia will produce urea.

Insufficient attention has been paid by many investigators of hydrolytic enzymes to the energy values and equilibria involved in hydrolysis. LEWIS and BURROWS (16) established the fact that the free energy value in aqueous solution at room temperature of ammonium cyanate is greater than that of

⁶ FEARON, W. R. The structure of urea with reference to its deamination and synthesis by urease. *Biochem. Jour.* 30: 1652-1660. 1936.

urea; likewise the free energy value of urea is greater than that of ammonium carbamate; *i.e.*, ammonium cyanate $\rightarrow$ urea $\rightarrow$ ammonium carbamate. If one assumes a 1 M. urea solution at room temperature and that no decomposition takes place to ammonium carbamate, it can be calculated that at equilibrium there will be 0.0067 M. ammonium cyanate and 0.9933 M. urea. But since urea is decomposing to ammonium carbamate, at equilibrium little or no cyanate will be present.

Any hypothesis which assumes that carbamate produces cyanate which in turn produces urea must in addition postulate an energy source for the synthesis of cyanate from carbamate.

Summary

1. The urease activity of *Soja max* has been determined by the use of histological and quantitative methods. The data for total urease are summarized in the graph (fig. 4), which shows the total urease content of the developing bean, and of the soy bean seedling throughout the life history of the plant. The distribution of urease in the various tissues of seedlings 8, 15, 31, and 52 days old is illustrated in a composite picture (figs. 1, 2). A description of the specific urease changes of the individual plant structures is given.

2. In the soy bean, as in the jack bean, the changes observed in urease content of the plant are due primarily to the changes in urease content of the parenchyma cells. Neither the cambium nor the cells derived from the cambium contain amounts of urease that can be detected with the indicator reagents.

3. The changes in urease distribution found in the jack bean and soy bean may be considered to hold for the Papilionaceae in general.

4. Analyses of urease, lipase, and protease in the developing soy bean indicate that the changes in lipase and protease activities do not follow the changes in urease activity.

5. Urease is considered a growth-bound enzyme. There is a rapid synthesis of urease in actively dividing cells. Synthesis continues during the stage of cell elongation, at the end of which period the urease content of the cell reaches a maximum. The urease content of the cell then begins to decrease to a certain level.

6. The changes in respiratory activity and urease activity at various stages of cell development follow similar courses.

7. It is suggested that by observing the changes of urease activity one may obtain an indication of the changes in the protoplasmic framework and soluble protein content of a cell.

8. The data on urease and respiratory activity are in accord with the interpretation that protoplasmic activity in the parenchyma cell continues

to increase until the cell has attained its maximum size; after that it decreases to a certain level as the cell becomes older.

9. The rôle of urease in the intermediary metabolism of the plant is discussed. From thermodynamic considerations it is calculated that any urea present in the plant cell will be converted by urease into ammonium carbamate. Ammonium carbamate, being unstable on the acid side of neutrality (the condition in the plant cell), will be hydrolyzed to ammonium carbonate. It is also shown that urease cannot function in the synthesis of urea from ammonium carbonate in the plant cell. The presence of large quantities of urease, especially in the meristematic or protein synthesizing regions of the jack bean and soy bean, suggests that urease performs the function of releasing ammonia from any free urea present and thus makes it available for protein synthesis.

10. There is no evidence that urease is transported, as such, from one portion of a plant to another.

The writer desires to express his gratitude for the many suggestions and kindly criticisms of Professor F. G. GUSTAFSON throughout the course of this investigation. Sincere thanks are due to Professor P. F. WEATHERILL of the Chemistry Department, University of Michigan, and to Professor H. B. BULL of the Agricultural Biochemistry Department, University of Minnesota, for their aid and advice in the thermodynamic calculations. The writer is also indebted to Professor C. M. WOODWORTH of the Illinois Experiment Station for supplying the soy beans for these experiments.

UNIVERSITY OF MICHIGAN
ANN ARBOR, MICHIGAN

LITERATURE CITED

1. BACH, D. Evolution de l'uréase dans les cultures de l'*Aspergillus niger*. Bull. Soc. Chim. Biol. **11**: 1007-1015. 1929.
2. BEZAGU, M. Variations de la respiration des cellules de feuille avec l'âge. Compt. Rend. Acad. Sci. (Paris) **169**: 701-702. 1919.
3. BLAGOWESTSCHENSKI, A. Untersuchungen über die Samenreifung. Biochem. Zeitschr. **157**: 201-219. 1925.
4. BORSOOK, H. Reversible and reversed enzymatic reactions. Ergebn. Enzymf. **4**: 1-38. 1935.
5. BURK, D. Azotase and Nitrogenase in *Azotobacter*. Ergebn. Enzymf. **3**: 23-56. 1934.
6. ETHERIDGE, W. C., HELM, C. A., and KING, B. M. A classification of soybeans. Missouri Agr. Exp. Sta. Bull. 131. 1929.
7. EULER, H. Chemie der Enzyme. II Teil 2nd. Abschnitt: 334-370. München. 1927.
8. FAURHOLT, C. Über ammonium carbonate, carbamate und carbonic acid. Zeitschr. anorg. Chem. **120**: 85-102. 1922.

9. FISCHER, E. Contributions to the study of the vegetable proteins. Biochem. Jour. **13**: 124-134. 1919.
10. GRANICK, S. Urease distribution in plants: general methods. Plant Physiol. **12**: 471-486. 1937.
11. ———. Urease distribution in *Canavalia ensiformis*. Plant Physiol. **12**: 601-623. 1937.
12. KIESEL, A., and TROITZKI. Beitrag zur Kenntnis der Verbreitung der Urease in Pflanzen. Zeitschr. physiol. Chem. **118**: 247-253. 1922.
13. KLEIN, G. Der Wandel des Stickstoffs in der grünen Pflanze. Ergebn. agr. Chem. **2**: 143-158. 1931.
14. ———, and TAUBÖCK, K. Harnstoff und Ureide bei den höheren Pflanzen. Biochem. Zeitschr. **241**: 413-459. 1931.
15. KREBS, H. A. Urea formation in the animal body. Ergebn. Enzymf. **3**: 247-264. 1934.
16. LEWIS, G. N., and BURROWS, G. H. Free energy of organic compounds. I. The reversible synthesis of urea and of ammonium cyanate. Jour. Amer. Chem. Soc. **34**: 1515-1529. 1912.
17. ———, and RANDALL, M. Thermodynamics and the free energy of chemical substances. New York. 1923.
18. LINDERSTRÖM-LANG, K., and HOLTER, H. Über die Peptidaseverteilung in Wurzel und Blattkeim des Malzkornes. Zeitschr. physiol. Chem. **204**: 15-53. 1932.
19. MACK, E., and VILLARS, D. S. Synthesis of urea with the enzyme urease. Action of urease in the decomposition of urea. Jour. Amer. Chem. Soc. **45**: 501-505, 505-510. 1923.
20. MURNEEK, A. E. Physiological rôle of asparagine and related substances in nitrogen metabolism of plants. Plant Physiol. **10**: 447-464. 1935.
21. ONSLOW, M. The principles of plant biochemistry. Cambridge Univ. Press. 1931.
22. SCHULZE, B., and SCHÜTZ, J. Die Stoffwandlungen in den Laubblättern des Baumes, insbesondere in ihren Beziehungen zum herbstlichen Blattfall. Landw. Versuchs-Sta. **71**: 299-352. 1909.
23. SEIFRIZ, W. The structure of protoplasm. Bot. Rev. **1**: 19-36. 1935.
24. SUMNER, J. Crystalline urease. Ergebn. Enzymf. **8**: 295-301. 1932.
25. TOVARNITZKY, V. Researches in the agricultural biochemistry of soya beans. Trudy Vses Nauchno-Issled Inst. Zernobob Kul'tur. **4**: 7-247. 1935.
26. VON OHLEN, F. A microchemical study of soybeans during germination. Amer. Jour. Bot. **18**: 30-49. 1931.

Radicle	604
Collet	604
Root	605
Hypocotyl	607
Stem	608
Epicotyl	609
Leaves	611
Plumule leaves	613
Flower and fruit	615
3. Urease distribution in the plant as a unit	618
4. Discussion	621
5. Summary	622
6. Literature cited	623

Part III. Urease distribution in *Sojox max.* Plant Physiology.
January, 1938.

1. Introduction	29
2. Urease distribution in various plant parts	29
3. Urease distribution in the plant as a unit	40
4. Discussion	
Comparison of urease distribution in Soy Bean and Jack Bean.....	42
Urease in other legumes	43
The distribution of urease, protease, and lipase in the developing and in the germinating seed	43
Urease and respiration	46
Urease and protoplasm	47
Urease and physiological age	48
Role of urease in intermediary metabolism	49
5. Summary	52
6. Literature cited	53
